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COMPARATIVE BIOCHEMISTRY

Volume II: Free Energy and Biological Function

COMPARATIVE BIOCHEMISTRY

A Comprehensive Treatise

Volume I: Sources of Free Energy
Volume II: Free Energy and Biological Function
Volume III: Constituents of Life—Part A
Volume IV: Constituents of Life—Part B
Volume V: Cells and Organisms—Part A
Volume VI: Cells and Organisms—Part B

COMPARATIVE BIOCHEMISTRY

A Comprehensive Treatise

Edited by

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Volume II

FREE ENERGY AND BIOLOGICAL FUNCTION

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PREFACE

The aim of this Treatise on Comparative Biochemistry is to provide a sound, critical, and provocative summary of present knowledge in the field. Although comparative biochemistry is the most recent approach to the study of biology, its domain is vast because it seeks to compare the physicochemical properties of every form of life. In this sense it includes all of biochemistry—not only those aspects common to life in general, but also the unique physicochemical manifestations which are characteristic of each of the several million species of living organisms comprising the phylogenetic scale. Biological diversity is inherently as interesting to comparative biochemistry as biological unity. Furthermore, comparative biochemistry is concerned not only with contemporary life, but with life of all ages reaching back to origins; it views evolution in terms of molecular rather than morphological changes.

Several excellent pioneer monographs on comparative biochemistry have been published but none is more than an introduction to the field. This certainly is no criticism, because the time and scholarship required by a single author to survey critically the whole subject would surely dwarf the effort expended by Darwin himself on the much more meager information which provided the foundations for the "Origin of Species" one hundred years ago. Consequently, each phase of Comparative Biochemistry has been written by an author critically selected for his competence in the subject with which he deals.

The organization of the Treatise has been of particular importance and concern because only systematic division of the field into well-related groups of subjects can provide a framework for comprehensive treatment of the whole subject with minimum overlap between chapters. In our concern for the integrity of the Treatise's organization and its utility to the reader, we have preferred to delay publication of individual volumes rather than have chapters appear out of their organizational context. However, when delay of this kind has occurred, every effort has been made by the Publishers to give authors the opportunity to incorporate the most recent information in their chapters.

The authors contributing to this Treatise have had a particularly difficult task. In most instances their chapters have been pioneering efforts. The chemists among them have had to approach their topics, in part, from the unfamiliar ground of comparative biology, while the biologists have had to consider molecular dynamics more closely than they might otherwise have done. In the effort to achieve over-all consistency of purpose and outlook, many writers have patiently revised their chapters. They all have had to brace themselves against the oversimplifying attractions of biochemical unity with the austerities of biochemical diversity.

In order to provide a systematic comparison of the biochemical phenomena of life throughout the phylogenetic scale, which has been our objective, the Treatise has been organized in the following manner: Volumes I and II are primarily concerned with the biological transformations of free energy. Volume I deals with the sources of biologically useful free energy, while Volume II describes how this free energy is utilized to support function. The structures of the principal classes of metabolites, their distributions, and the comparative enzymology of their biogenesis and metabolism are the subjects of Volumes III and IV. The comparative biochemistry of organized reactions systems and the biological functions dependent upon these systems are discussed in Volumes V and VI.

The Editors were saddened by the deaths of three distinguished contributors, Professor E. Newton Harvey of Princeton University, Professor C. Fromageot of the University of Paris, and Professor W. Bergmann of Yale University. They possessed unique commands of their fields of interest, and we are fortunate to be able to include their chapters on comparative aspects of bioluminescence, inorganic metabolism, and steroid distribution.

We wish to record our gratitude to the staff of our Publishers, Academic Press, for warm support and professional assistance throughout the preparation of the Treatise. They have made the task of editing far less onerous than it might have been. One of us (H. S. M.) wishes to thank Professor Sir Alexander Todd for the hospitality of his Department at the University of Cambridge during the editing of Volumes I and II, and Professor E. S. West of Portland, and Mr. T. N. Norris, M.A., of Cottenham, Cambs., for helpful editorial suggestions.

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February, 1960

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A Comprehensive Treatise

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* Tentative contents.

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[*] Most of the names refer to phyla, except in a few cases where some of the smaller taxonomic groups are shown. Capitalized names written across lines are groups including all forms above the name.

NOTE: Charts I, II, and III were prepared by Helen A. Stafford, Reed College, Portland, Oregon. For further information see "A Guide to the Nomenclature and Classification of Organisms," by Dr. Stafford, in Vol. I of this treatise.

CHART I
HYPOTHETICAL PHYLOGENETIC RELATIONSHIPS BETWEEN EXTANT MAJOR GROUPS OF ORGANISMS*

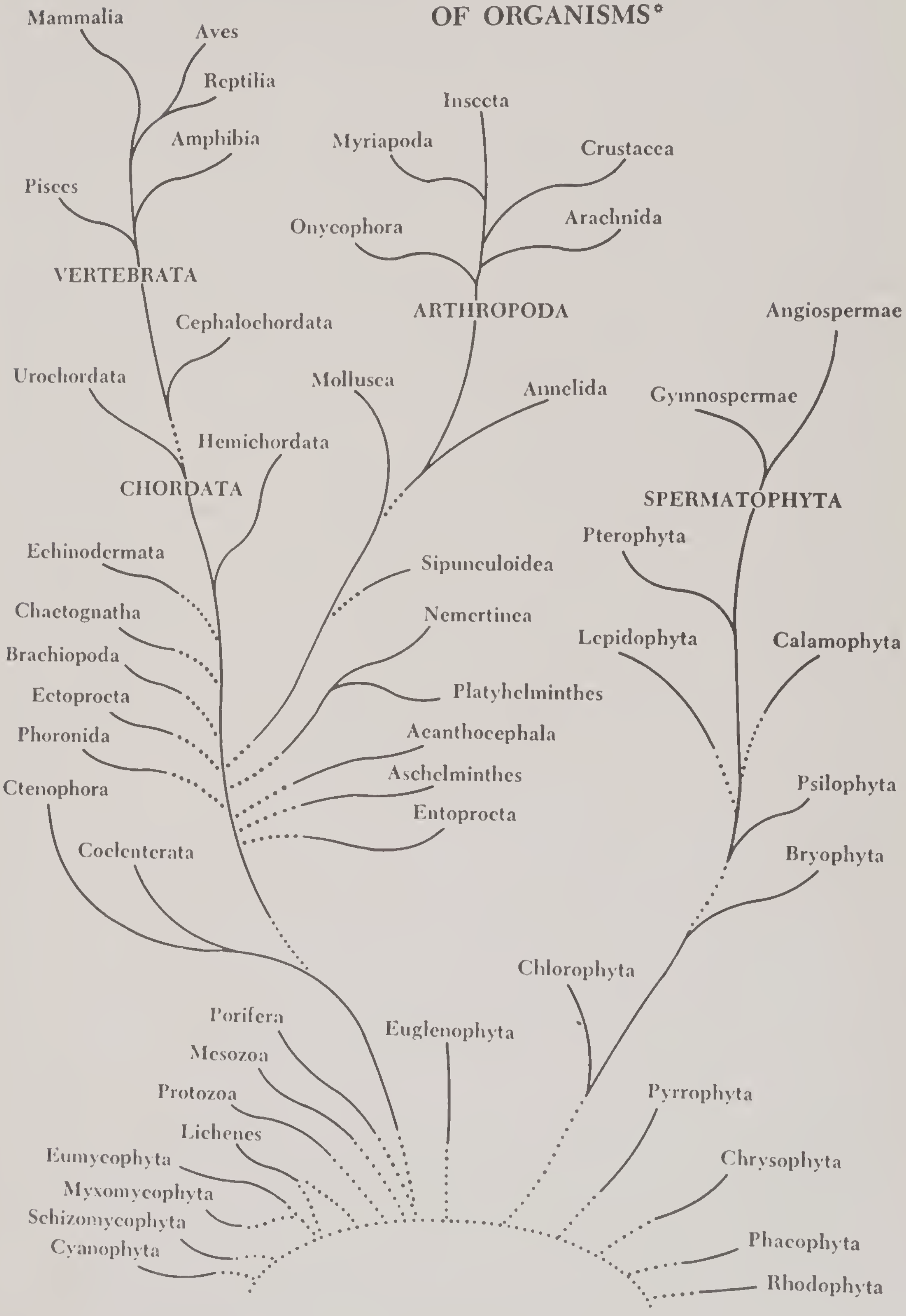

CHART II: ANIMAL KINGDOM

Divisions	Estimated Number of Species[d]	Taxonomic Classifications			
Protozoa (acellular animals)	15,000				
Mesozoa	–				
Porifera (sponges)	5,000				
Coelenterata (coelenterates)	10,000				Radiata
Ctenophora (comb jellies)	100				
Platyhelminthes (flat worms)	6,000	Acoelomates		Protostomia	Bilateria
Nemertinea (nemertine worms)	500				
Aschelminthes[a] Acanthocephala[a]	7,000	Pseudocoelomates			
Entoprocta[b] Ectoprocta[b] (moss animals)	3,000	Eucoelomates	Schizocoela		
Phoronida	15				
Brachiopoda (lamp shells)	120				
Mollusca (mollusks)	70,000				
Sipunculoidea	–				
Annelida[c] (segmented worms)	6,500				
Arthropoda (arthropods)	750,000				
Chaetognatha (arrow worms)	30		Enterocoela	Deuterostomia	
Echinodermata (echinoderms)	5,000				
Hemichordata Chordata (including vertebrates)	60,000				

---

[a] Includes Rotifera, Gastrotricha, Kinorhyncha, Nematoda, Nematomorpha, Priapuloidea. Formerly called Nemathelminthes.

[b] Formerly in Bryozoa.

[c] Includes Echiuroidea.

[d] Taken from "Handbook of Biological Data" (*4*), p. 533.

CHART III: PLANT KINGDOM

Divisions	Estimated Number of Species[d]	Major Synonymous Terms		
Euglenophyta (euglenoids)	340	**Algae**	**Thallophyta**	**Cryptogamia**
Chlorophyta (green algae)	5,700			
Pyrrophyta (cryptomonads, dinoflagellates)	1,000			
Chrysophyta (yellow green algae, diatoms)	5,700			
Phaeophyta (brown algae)	900			
Rhodophyta (red algae)	2,500			
Cyanophyta[a] (blue-green algae)	1,400			
Schizomycophyta[a] (bacteria)	1,300[e]	**Fungi**		
Myxomycophyta (slime molds)	430			
Eumycophyta (true fungi)	74,000			
Lichenes (lichens)	15,500			
Bryophyta (mosses and liverworts)	23,800		**Bryophyta**	
Psilophyta[b] (whisk ferns)	3	**Psilopsida**	**Tracheophyta**	
Calamophyta[b] (horsetails)	30	**Sphenopsida**		
Lepidophyta[b] (lycopods)	1,300	**Lycopsida**		
Pterophyta[b, c] (ferns)	10,000	**Pteropsida**		
Spermatophyta (seed plants)	201,000			**Phanerogamia**

---

[a] Sometimes grouped as Schizophyta.

[b] Formerly classed as Pteridophyta.

[c] Formerly classed as Filicineae in Pteropsida.

[d] Taken from "Handbook of Biological Data" (*4*), p. 533.

[e] There is much disagreement concerning designation of species here.

CHAPTER 1

Free Energy and the Biosynthesis of Phosphates

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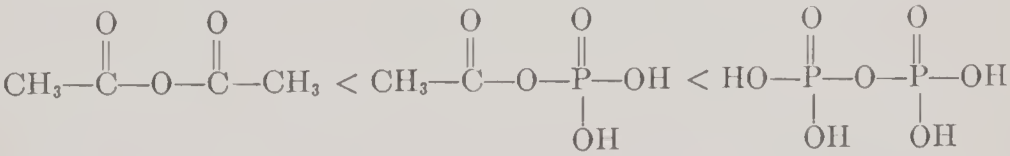
I. Introduction

Liebig (*1*) directed attention to the importance of phosphorus as a major component of tissues, and urged the use of phosphates as plant fertilizers. Thus he anticipated the later work of Büchner (*2, 3*) and of Harden and Young (*3, 4*), who laid the foundations of present knowledge of phosphorus metabolism. In both the studies of Büchner and of Harden and Young cell-free extracts of bakers' yeast were used and it was found that formation of alcohol during anaerobic fermentation was dependent on the supply of inorganic phosphate, which was converted into organic phosphate. These findings led to extensive investigation of phosphate esters in living things and, although they were first thought to be unimportant side products, it was soon clear that these compounds were essential intermediates in metabolic transformations (*3*).

Though many biochemical reactions are now known in which phosphates are not involved, the importance of these compounds as activated metabolites cannot be overstressed. Phosphorylated compounds are invariably associated with the energy utilization fundamental to life. The sources of energy for living organisms are extremely diverse, but in all cases (even among autotrophs) the energy is obtained by oxidation of some primary compound. Exergonic (energy-producing) oxidative reactions are coupled with endergonic syntheses of organic phosphates from inorganic phosphate. Subsequent syntheses of new cell material are coupled with utilization of these organic phosphates. The nature and biosynthesis of these compounds and thermodynamic aspects of their metabolism will be discussed in this chapter.

It is not obvious why phosphate esters rather than esters of other inorganic acids predominate in biological systems. Neither sulfates nor arsenates have comparable importance; the latter compounds are usually toxic to living cells. It is probably significant that phosphate anhydrides combine high activation energies of nonenzymatic hydrolysis with large negative free energies of hydrolysis. This permits controlled enzymatic cleavage of the anhydride rather than spontaneous hydrolysis. This stabilizing effect of phosphate is well illustrated by the increasing resistance to hydrolysis in the series of compounds:*



$$CH_3{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}CH_3 < CH_3{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle O}{\|}}{P}}{-}OH < HO{-}\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle O}{\|}}{P}}{-}O{-}\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle O}{\|}}{P}}{-}OH$$

Lipmann (5) has pointed out that acetic anhydride is hydrolyzed rapidly in neutral conditions, acetyl phosphate is slightly more stable, and pyrophosphate is quite resistant to hydrolysis at pH 7. Phosphates are probably protected from hydroxyl attack by their negative charge. Dissociation constants of phosphates are such that a change of pH in the physiological range alters their charge, and thus modifies their interaction with enzymes. This provides a control mechanism by which cells may adjust reaction rates in response to changes in environment.

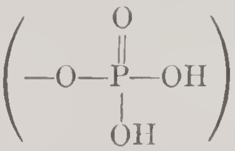
II. Thermodynamics of Phosphoryl and Phosphate Transfer Reactions

A. Free Energy and Equilibria

Almost all organic phosphorus compounds in living organisms are phosphate esters, amides, or anhydrides. They are obtained by transfer

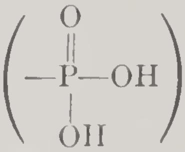
[*] In general, structural formulas show the undissociated forms of molecules. The abbreviations used in this chapter are listed at the end.

reactions initially from inorganic phosphates to a limited number of acceptors. The reactive phosphates so formed subsequently phosphorylate a large number of other acceptors thus giving the numerous important metabolites which contain phosphorus. Details of mechanism are discussed later and in this section "phosphate transfer" is used to describe both transfer of phosphate and phosphoryl groups.



$$\left(-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-OH\right)$$

Phosphate group



$$\left(-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-OH\right)$$

Phosphoryl group

Thermodynamic information about phosphate donors and acceptors provides a useful classification of these compounds and their reactions, particularly as it is independent of the detailed mechanisms involved in phosphate transfers. From thermodynamic data it is possible to predict the equilibrium composition of a system of reactants and the maximum work which can be done in conjunction with a phosphate transfer. The thermodynamic function which is most useful in describing these characteristics of a system of reactants is the free energy of reaction, ΔF, which is the increment of free energy for the reaction under specific conditions [see Lewis and Randall (*6*)].* Free energy, F, and its relationship to internal energy, E, enthalpy, H, and entropy, S, have been defined elsewhere in this treatise (cf. Vol. I, Chapter 2). Some biochemical aspects of free-energy changes have been discussed by Pardee (*7*). For a reaction at temperature T

$$\Delta F = \Delta H - T\Delta S.$$

At constant pressure, p, if there is a volume change, Δv, during the reaction, then

$$\Delta F = \Delta E + p\Delta v - T\Delta S.$$

F is also known as the thermodynamic potential. In an isothermal system at constant pressure any change is accompanied by a decrease in free energy, so that at equilibrium F has a minimum value. The difference in free energy between a system in its actual state and its equilibrium state is a measure of its tendency to approach the equilibrium state. The thermodynamic equilibrium constant, K_a, is the product of the equilibrium activities of reactants on the right-hand side in a chemical equation, divided by the corresponding product of the left-hand side reactants. The free energy of reaction and the equilibrium constant are related as follows:

---

* The symbols F and G are both commonly used to represent the Gibbs free energy function and they are interchangeable. The symbol F has been used throughout this volume.

$$\Delta F^\circ = -RT \ln K_a,$$

where ΔF° is the free energy of reaction in standard conditions (in calories), R is the gas constant (1.987 cal./degree/mole, where F° is in calories), and T is the absolute temperature. Most information about free energies of phosphate transfer has been obtained by applying this equation to equilibrium concentrations determined after enzymatic catalysis. In most cases it has been possible to measure equilibrium concentrations in such dilute solutions that they are not significantly different from the corresponding activities. From such measurements, free energies have been calculated from the relationship

$$\Delta F^\circ = -RT \ln K_c,$$

where K_c is the equilibrium constant based on concentrations instead of activities. In some cases very high concentrations of one or more reactant have been used in order to obtain enough of the minor component to measure (*8*), so that the difference between observed concentrations and the corresponding activities may be great enough to cause a significant error in the value of ΔF°. An excellent discussion of this and other aspects of free-energy measurements has been given by Burton and Krebs (*9*). Measurement of radioactivity of components in low concentration at equilibrium with labeled reactants has enabled Ginodman (*10*) to calculate the equilibrium constant of the reaction

$$\text{Glucose} + \text{P}_i \rightleftarrows \text{Glucose-6-phosphate} + \text{H}_2\text{O}$$

at glucose concentrations as low as 0.01 M. This method will certainly improve the accuracy of estimates where K_a is far removed from unity and thus permit the evaluation of numerically large ΔF° from equilibrium data.

Most of the equilibria discussed in this chapter have been established by enzymatic catalysis, and are often not true equilibria, since some reactants have not been in equilibrium with water. For example, in the reaction catalyzed by adenylate kinase,

$$2\text{ADP} \rightleftarrows \text{ATP} + \text{AMP}$$

useful equilibrium and free-energy data may be calculated though none of the reactants are in equilibrium with water. The nonenzymatic hydrolyses are usually so much slower than the enzymatic reaction that no significant error arises in calculating ΔF from the "pseudoequilibrium" composition when no further measurable enzymatic change is taking place.

If H^+ or OH^- is a reactant, ΔF° refers to the equilibrium with these ions at unit activity, and hence is not directly applicable to physiological conditions. The quantity $\Delta F'$, which is the free-energy change for a reaction at a specified pH with all reactants other than H^+ at unit activity is often used. If H^+ or OH^- are not reactants, ΔF° equals $\Delta F'$. A number of standard free-energy changes at pH 7 and 25° (or at other pH values and temperatures where indicated) have been collected in Table I.* Where equilibrium values for a particular reaction have not been determined, free energies of hydrolysis have been estimated by combination of data for successive reactions. Thus in estimating the $\Delta F'$ of hydrolysis of ATP, free-energy changes for the reactions

$$\alpha\text{-D-Galactose-1-phosphate} + H_2O \rightleftarrows (\alpha + \beta)\text{-D-Galactose} + P_i \qquad (\Delta F', -5.0 \text{ kcal./mole})\dagger$$

and

$$(\alpha + \beta)\text{-D-Galactose} + \text{ATP} \rightleftarrows \alpha\text{-D-Galactose-1-phosphate} + \text{ADP} \qquad (\Delta F', -1.9 \text{ kcal./mole})\dagger$$

have been combined to give, by summation

$$\text{ATP} + H_2O \rightleftarrows \text{ADP} + P_i$$

for which $\Delta F'$ is -6.9 kcal./mole.†

Conversely, if the $\Delta F'$ of reaction of two phosphates with a common phosphate acceptor (usually water) is known, it is possible to predict the $\Delta F'$ of reaction between the two phosphates, and from this the equilibrium which results when they are reactants. Thus, when arranged in order of their $-\Delta F'$ of hydrolysis, organic phosphates are then in a sequence of thermodynamic reactivities. Table II lists this sequence for some important metabolites. Klotz (*80*) has very aptly named this quantity the "phosphate transfer potential." This term avoids many objectionable aspects of the expression "high-energy (or energy-rich) phosphate bond" which is often applied to compounds with large $-\Delta F'$ of hydrolysis. Lipmann (*81*) referred to phosphate groups with large negative free energies of hydrolysis as having high "group potentials," and it seems unfortunate that this nomenclature, which is consistent with the definition of F as the thermodynamic potential, has not been more widely used (see also Gillespie *et al.*, *82*). Since a phosphoryl group is transferred in these reactions, the term "phosphoryl transfer potential" seems preferable to "phosphate transfer potential" and has been used in the following sections.

Though ΔF is the maximum work which can be done in conjunction with a chemical reaction, the actual work done is always less, since the changes take place at a finite rate and not in a thermodynamically reversible manner. If, as Gillespie *et al.* (*82*) suggest, $\Delta F'$ is thought of as a modified equilibrium constant its most useful aspects are less likely to be obscured. Though the difference between the phosphate transfer potentials of "high-energy" and "low-energy" phosphates is smaller than early evidence indicated (see Table II), this classification, which was proposed and developed so effectively by Lipmann (*81, 83*), has been a central concept in the study of intermediary metabolism during the past decade (*15, 84–89*) and is certain to have permanent value.

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* References *8–79* are in Table I. See p. 14.

† See Table I, 17c,A and 16a,B; also reference *29*.

B. Factors Influencing the Free Energy Change of Reactions

1. *Concentration of Reactants and pH*

Standard free energies of reaction are defined for unit activities of reactants. Under physiological conditions, reactants are normally at much lower activities.

There is a simple relationship between the free energy change of a reaction and the activities of the reactants. In general, for the reaction

$$bB + cC + \cdots \rightleftarrows mM + nN + \cdots$$

if $[a_B]$, $[a_C]$, etc. are the activities, and b, c, are the number of molecules of reactants B, C, etc., and

$$J = \frac{[a_M]^m[a_N]^n \ \cdots}{[a_B]^b[a_C]^c \ \cdots}$$

then the actual free-energy change is

$$\Delta F = \Delta F^\circ + RT \ln J.$$

For example, if H^+ is a product in a hydrolysis, such as

$$ATP^{4-} + H_2O \rightleftarrows ADP^{3-} + P_i^{2-} + H^+$$

ΔF°, which refers to unit activity of H^+ will differ from $\Delta F'$, the free energy change at a specified pH. The relationship can be derived from the general expression given above. Thus

$$\begin{aligned}\Delta F' &= \Delta F^\circ + RT \ln [a_{H^+}]/1 \\ &= \Delta F^\circ - 2.3\ RT\ (\text{pH}).\end{aligned}$$

If this reaction takes place in physiological conditions, where the concentrations of ATP, ADP, and P_i are not significantly different from the activities, then

$$\Delta F = \Delta F^\circ + RT \ln \frac{(\text{ADP})(P_i)}{(\text{ATP})(H_2O)} - 2.3\ RT\ (\text{pH}).$$

Thus if (P_i) is $10^{-2}\,M$ and the concentrations of ADP and ATP are equal

$$\begin{aligned} \Delta F - \Delta F' &= RT \ln 10^{-2} \\ &= 1.97 \times 310 \times 2.3 \log_{10} (10^{-2})\text{cal./mole at } 37^\circ \\ &= -2.8 \text{ kcal./mole,} \end{aligned}$$

and

$$\begin{aligned} \Delta F &= (-6.9 - 2.8) \text{ kcal./mole (cf. Table II)} \\ &= -9.7 \text{ kcal./mole.} \end{aligned}$$

In this case the reason for the greater magnitude of ΔF in dilute solution is that one of the reactants, water, is at unit activity. Clearly, if any pair of reactants on opposite sides of a chemical equation differ in concentration by a factor of 10, the actual free-energy change will differ from the apparent free-energy change by 2.3 $RT \log_{10} 10$ (i.e., by about 1.4 kcal./mole). In studies of cellular energetics, information is required about free-energy changes of reactions taking place within cells, and frequently in multiphase regions such as mitochondria and the endoplasmic reticulum. At present there is no available information about the concentrations of reactants at enzyme surfaces in such systems. Moreover, the reactants themselves probably never reach equilibrium, and may differ very considerably in concentration. In calculating the thermodynamic efficiency of a metabolic process, free-energy changes at physiological rather than at standard concentrations must be considered. Krebs and Kornberg (*90*) have given a valuable discussion on this point (*35*).

When free energies are calculated from equilibrium concentrations, the concentration of each reactant which is taken is the sum of the concentrations of all ionic species of that reactant at the pH involved. Free energies of reaction therefore include the free energies of ionization of the reactants, and as these are dependent on pH, the free energy of reaction is also a function of pH. This is well illustrated in Oesper's (*15*) discussion of equilibrium data for the reaction

$$\underset{(\text{p}K\ 1.4 \text{ and } 6.4)}{\text{Glycerol-1-phosphate}} + H_2O \rightleftarrows \underset{(\text{p}K\ 2.0 \text{ and } 6.8)}{\text{Glycerol} + P_i}$$

Since successive pK values are well separated, only one dissociation is incomplete at any pH.*

The free energy of ionization of any one reactant to form an equilibrium mixture at the pH considered is

$$\Delta F_i = 2.3\,RT \sum (\text{p}K_i - \text{pH}) - RT \ln [1 + (H^+)/K]$$

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* When more than one dissociation is incomplete the equations are more complicated [cf. Dixon (*84*) and Alberty *et al.* (*91*)].

where summation is over all dissociations of that reactant, and the last term allows for incompleteness of the last ionization. Oesper (*15*) has calculated the ratio of equilibrium constants for glycerophosphate hydrolysis at pH 5.8 and pH 8.5. At pH 5.8 the difference of the ΔF_i of products and reactants is

$$2.3\,RT\,[(2.0-5.8)+(6.8-5.8)] - RT\ln\left(1+\frac{10^{-5.8}}{10^{-6.8}}\right)$$
$$-2.3\,RT\,[(1.4-5.8)+(6.4-5.8)] + RT\ln\left(1+\frac{10^{-5.8}}{10^{-6.4}}\right),$$

i.e., $2.3\,RT\,[(2.0-5.8)+(6.8-5.8)-\log(1+10)-(1.4-5.8)$
$\qquad -(6.4-5.8)+\log(1+4)]$.

At pH 8.5 both ionizations are complete and the difference between ΔF_i of reactants and products is

$$2.3\,RT\,[(2.0-8.5)+(6.8-8.5)-(1.4-8.5)-(6.4-8.5)]$$

and the net change in ΔF_i between pH 5.8 and 8.5 is

$$\begin{aligned}(\Delta F_{i(\mathrm{pH}8.5)} - \Delta F_{i(\mathrm{pH}5.8)}) &= 2.3\,RT\,[\log(1+10)-\log(1+4)]\\ &= 2.3\,RT\log(11/5)\\ &= 0.49\ \text{kcal./mole at } 37^\circ.\end{aligned}$$

Thus it can be predicted that

$$K_{c(\mathrm{pH}\ 5.8)}/K_{c(\mathrm{pH}\ 8.5)} = 11/5,\ \text{i.e., } 2.2.$$

Meyerhof and Green (*8*) found $K_{c(\mathrm{pH}\ 5.8)} = 4.3$,
and $K_{c(\mathrm{pH}\ 8.5)} = 2.0$,

$$\text{i.e., } K_{c(\mathrm{pH}\ 5.8)}/K_{c(\mathrm{pH}\ 8.5)} = 2.2.$$

As pointed out by Oesper (*15*), when there are the same number of ionizing groups in the reactants and products the pH and p*K* terms cancel out, leaving only the ln $(1+(\mathrm{H}^+)/K)$ terms, which allow for incomplete ionization. When a new ionizing group is formed during the reaction a new term of the form 2.3 *RT* (p*K* — pH) appears in the free energy of hydrolysis, and although the p*K* terms will cancel out on calculation of the difference in ΔF of hydrolysis at the two pH values, (pH_1 and pH_2), the term 2.3 *RT* ($\mathrm{pH}_2 - \mathrm{pH}_1$) will remain. This behavior was illustrated above in discussing the effect of concentration on the free energy of hydrolysis of ATP. Podolsky and Morales (*32*) have discussed the effect of ionization in this reaction. Burton and Krebs (*9*) have also pointed out the advantage of calculating free energy data for pH 7.5, where many biologically important compounds exist mainly in a single ionic form.

2. *Complex Formation*

Analytical methods used in equilibrium studies not only measure the sum of the concentrations of all ionic species of a reactant, but also the concentrations of any complexes of the reactant which are present. This introduces special problems in calculating the free energy changes of reactions involving phosphates. Almost invariably, these form complexes with metals, especially magnesium, which are always present under physiological conditions (*92*). Free energies of metal-complex formation by both reactants and products will be included in the calculated free-energy change of a reaction. Just as free energies of ionization depend on pH, so these free energies of complex formation depend on metal ion concentrations. When free-energy changes calculated from equilibrium data are applied to systems with different magnesium concentrations they will be in error by an amount which depends on the different extent of complex formation in these systems.

Robbins and Boyer (*22*) have measured the effect of Mg^{2+} on K_c of the reaction

$$\text{ATP} + \text{Glucose} \rightleftarrows \text{ADP} + \text{Glucose-6-phosphate}$$

Burton and Krebs (*9*) have estimated the proportions of ATP and ADP which exist as magnesium complexes in several equilibria of the glycolytic reactions. Noda *et al.* (*59*) have examined this problem in detail for the creatine kinase equilibrium

$$\text{Creatine phosphate} + \text{ADP} \rightleftarrows \text{Creatine} + \text{ATP}$$

The apparent equilibrium constant

$$(\text{ATP})(\text{creatine})/(\text{ADP})(\text{creatine phosphate})$$

at any pH was found to depend on the concentration of magnesium ion. At high concentrations of magnesium salt the apparent equilibrium constant was only slightly dependent on the concentrations of reactants. Over the range 0.006–0.02 M Mg^{2+}, at pH 8.9, the equilibrium constant varied from 2.8 to 5.2, but at 0.001 M Mg^{2+} the apparent equilibrium constant was less than 1. Thus at this pH, for certain concentrations of reactants, the phosphoryl transfer potential of creatine phosphate is greater than that of ATP at high Mg^{2+} concentrations, but less than that of ATP at low Mg^{2+} concentrations. This effect, which has also been observed with the arginine kinase equilibrium (*56*), may be of considerable physiological significance.

The influence of Mg^{2+} on equilibria is not confined to reactions involving phosphorylated compounds. Stern (*93*) reports that the equilibrium constant for the reaction

$$\text{Succinyl-S-CoA} + \text{Acetoacetate} \rightleftarrows \text{Acetoacetyl-S-CoA} + \text{Succinate}$$

is dependent on both H^+ and Mg^{2+} concentration.

3. *Temperature*

The variation of ΔF with T is expressed by the Gibbs-Helmholtz equation [cf. Klotz (*94*)]:

$$\left(\frac{\partial(\Delta F/T)}{\partial T}\right)_P = \frac{-\Delta H}{T^2}$$

The use of this equation may be illustrated by comparing the free energies of hydrolysis of ATP at 25° and 40°.

Taking $\Delta F'$ as -6.9 kcal./mole at 25° for ATP hydrolysis (Table II) and the heat of hydrolysis, ΔH, as -4.8 kcal./mole for this reaction in the range 20–40°,

$$\int d\,\frac{\Delta F'}{T} = -\int \frac{\Delta H}{T^2}\,dT$$

i.e., $$\frac{\Delta F'}{T} = \frac{\Delta H}{T} + C \text{ where } C \text{ is a constant.}$$

$$\Delta F' = \Delta H + CT$$

i.e., $$-6.9 = -4.8 + 298\,C$$

and $$C = -2.1/298.$$

At 40°, $$\Delta F' = -4.8 + \frac{-2.1}{298} \times 313 \text{ kcal./mole}$$

$$= -7.0 \text{ kcal./mole.}$$

The change in free energy of hydrolysis is -0.1 kcal./mole. Since the physiological temperature range is small (270–320°K) it is clear that the influence of temperature on group transfer potentials is almost negligible.

C. CORRELATION OF PHOSPHORYL TRANSFER POTENTIAL WITH CHEMICAL STRUCTURE

1. *Phosphates of Simple Alcohols*

The high concentration of water (about 55 M) at unit activity lowers $\Delta F'$ of hydrolysis by $RT \ln 55$ (about 2.2 kcal./mole) below the value which would be obtained if water, like the other reactants, was present at 1 M concentration. Thus simple phosphate esters such as glycerol-1-phosphate, for which the free energy of hydrolysis at pH 7 (i.e., $\Delta F'$) is -2.1 kcal./mole, and glucose-6-phosphate, for which $\Delta F'$ is -3.3 kcal./mole, would have very small free energies of hydrolysis with an equimolar concentration of water. The greater transfer potentials of other phosphates may be attributed to such factors as: (*a*) electrostatic repulsion between anionic components or products of hydrolysis; (*b*) increased resonance and ionization energies after hydrolysis; and (*c*) isomerization of the compound formed by removal of the phosphate group. The quantitative importance of these effects has not yet been accurately determined. A number of authors (*15, 81, 86, 95–97*) have discussed the relationship between structure and transfer potential. Some of the metabolites which show the influence of these effects are discussed below.

2. *Hemiacetal Phosphates*

a. α-D-Glucose-1-phosphate. This ester has a higher phosphoryl transfer potential ($\Delta F'$ of hydrolysis, -5.0 kcal./mole) than the corresponding alcoholic ester, glucose-6-phosphate. On hydrolysis the sugar is free to form an equilibrium mixture of its isomers, and the free energies of isomerization will contribute to the free energy of hydrolysis.

b. α-D-Galactose-1-phosphate. Free energy of hydrolysis of this ester is about -5 kcal./mole. The phosphoryl transfer potentials of galactose-1-phosphate and glucose-1-phosphate are probably identical (Table I). The free-energy change of the transformation:

$$\alpha\text{-D-Galactose-1-phosphate} \rightarrow \alpha\text{-D-Glucose-1-phosphate} \qquad (\Delta F' = -0.7 \text{ kcal./mole})$$

corresponds to that of the conversion of an axial-OH at C-4 of galactose into an equatorial-OH at C-4 of glucose (about -0.8 ± 0.2 kcal./mole) (*27, 28*). Since the standard free-energy change of the reaction:

$$\text{ATP} + (\alpha + \beta)\text{-D-Galactose} \rightarrow \text{ADP} + \alpha\text{-D-Galactose-1-phosphate}$$

at pH 7.0 and 25° is -1.9 kcal./mole in the presence of 0.025 M Mg^{2+}, the phosphoryl transfer potential of adenosine triphosphate is 6.9 kcal./mole (*29*). This is in close agreement with the value 6.9 ± 0.3 kcal./mole calculated for these conditions from other equilibrium data (*98*).

3. *Pyrophosphates*

Hill and Morales (*97*) suggest that electrostatic repulsion between anionic groups on each side of the P—O—P bond of pyrophosphates contributes -5 kcal./mole to the $\Delta F'$ of hydrolysis. It is also likely that the sum of the resonance energies of the mesomeric products formed on hydrolysis is greater than the resonance energy of ATP before hydrolysis of the phosphoanhydride. Both effects exist in the hydrolysis of all pyrophosphates. Delocalization of electrons in the vicinity of the anhydride —O— link is less complete than in the ionized products (*96*).

a. *Adenosine diphosphate and adenosine triphosphate.* The free energy of hydrolysis of ATP has been measured in many ways, and a number of values are shown in Table I. Earlier estimates (about —10 to —14 kcal./mole at pH 7) based on approximate thermal data are now known to be of too great a magnitude. From available information the most reasonable value seems to be —6.9 ± 0.2 kcal./mole at pH 7 and 25° in the presence of Mg^{2+}. It is 0.5 kcal./mole more negative than the free energy of hydrolysis of the terminal phosphate of ADP and 1 kcal./mole less negative than that of the inner phosphoanhydride bond of ATP. These findings are consistent with the configuration of ATP shown in Fig. 1. Szent-Györgyi (*99*) has suggested that the terminal phosphates of ATP are bound to the amino group and to N-7 of the adenine ring, either directly or through formation of a magnesium complex. If this is the case, repulsion between the anionic middle and terminal phosphates would be decreased and would result in a less negative $\Delta F'$ of hydrolysis of the terminal bond than of the inner anhydride bond. These steric relationships (*100*) can be seen from molecular models and are supported by rotatory dispersion data (*101*).

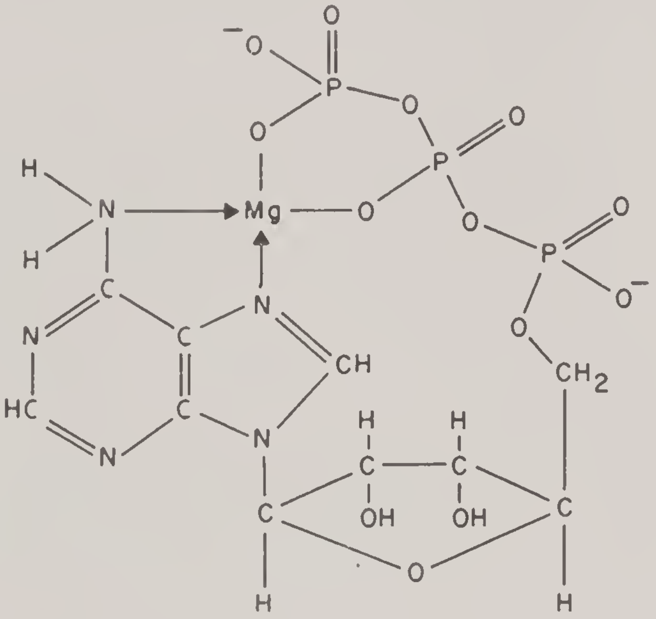

FIG. 1. Possible structure of adenosine triphosphate-magnesium complex.

Epp and associates (*102*) have some evidence from infrared spectra that magnesium complexes of both ATP and ADP may have this structure, which is also consistent with recent observations the metal complexes of adenine (*103*).

Since a new ionizing group is liberated on hydrolysis of ATP, the free energy of ionization contributes substantially to the total free energy of hydrolysis.

b. *Inosine triphosphate.* Since the equilibrium constant of the reaction

$$\mathrm{ATP} + \mathrm{IDP} \rightleftarrows \mathrm{ADP} + \mathrm{ITP}$$

is approximately 1 at pH 6 and 0.01 M Mg^{2+} concentration,* the group transfer potential of the terminal phosphate of inosine triphosphate is the same as that of ATP in these conditions. Here also, magnesium complex formation is possible.

c. *Uridine diphosphate and uridine triphosphate.* There is no significant free-energy change in the reaction

$$\mathrm{UTP} + \mathrm{UMP} \rightleftarrows 2\mathrm{UDP}$$

Phosphoryl transfer potentials of the terminal phosphates of UDP and UTP are therefore the same.* This contrast with the adenyl nucleotides may be due to decreased intramolecular complex formation, of the type discussed above, which is possible in pyrimidine nucleotides.

d. *Inorganic pyrophosphate.* The $\Delta F'$ of hydrolysis of inorganic pyrophosphate is probably about —6.7 kcal./mole.* Ging and Sturtevant (*42*) found that the heat of hydrolysis (ΔH) of inorganic pyrophosphate is —5.8 kcal./mole, as compared with —4.8 kcal./mole (*32*) for hydrolysis of the terminal phosphate of ATP. If the entropy changes in both reactions were similar, ΔF for hydrolysis of pyrophosphate would be about 1 kcal./mole more negative than that of the terminal phosphate of ATP. The entropy change on hydrolysis of pyrophosphate is probably less than that on hydrolysis of ATP at its terminal anhydride.

4. *Guanidine Phosphates*

a. *Creatine phosphate.* The free energy of hydrolysis of creatine phosphate can be calculated from the equilibrium constant of the reaction catalyzed by creatine kinase

$$\mathrm{Creatine}^{\pm}\text{-phosphate}^{2-} + \mathrm{ADP}^{3-} + \mathrm{H}^{+} \rightleftarrows \mathrm{Creatine}^{\pm} + \mathrm{ATP}^{4-}$$

In this reaction, in contrast to that of ATP hydrolysis, the free-energy change becomes more positive at higher pH values. This increase balances a corresponding decrease in the free energy of hydrolysis of ATP ($\Delta F'$, $-7.4 - 2.3\,RT\,(\mathrm{pH} - 7.5)$ kcal./mole between pH 7.5 and pH 10). Therefore in this range, where all reactants have the ionic forms shown, the free energy of hydrolysis of creatine phosphate should be constant.

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* See Table I.

TABLE I

FREE ENERGIES OF PHOSPHATE TRANSFER AND RELATED REACTIONS

Reaction		T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'^a$	References
1. Glycerol-1-phosphate + H_2O ⇵ Glycerol + P_i	A	37.5	8.4	?	0.75	−2.3	(*11, 12*)
	B	20	5.8	?	1.82	−2.7	(*13*)
	C	38	8.5	?	0.7	−2.2	(*8*)
	D	38	5.8	?	1.5	−2.7	(*8*)
	E	37	6.7	?	0.72	−2.3	(*10*)
	F	37	7.5	?	0.96	−2.5	(*10*)
	G	37	7.8	?	0.84	−2.4	(*10*)
	H	37	8.3	?	0.43	−2.0	(*10*)
	I	37	8.8	?	0.65	−2.2	(*10*)
	J	25	7.0	?	0.63	−2.1	(*10*)
2. Glycerate-3-phosphate + H_2O ⇵ Glycerate + P_i	A	30	8.5	?	?	−3.0	(*14, 15*)
3. Glycerate-2-phosphate ⇵ Glycerate-3-phosphate	A	24	?	?	6.5	−1.1	(*9, 14*)
	B	—	—	—	—	−0.8	(*16*), from (*14*)
	C	30	4.6–6.65	?	6.3	−1.1	(*17*)
4. Glycerate-2-phosphate + H_2O ⇵ Glycerate + P_i	A	38	8.5	—	—	−4.1	(*15*) [From 2A (−3.0) and 3A (−1.1)]
5. Glucose-6-phosphate + H_2O ⇵ Glucose + P_i	A	38	8.5	?	2.0	−2.9	(*8*)

	B	38	5.8	?	4.3	−3.4	*(8)*
	C	25	7.0	0.002–0.005	4.8	−3.3	*(29)*
	D	37	8.2	?	1.00	−2.5	*(10)*
	E	25	7.0	?	1.48	−2.6	*(10)*
	F	37	7.4	?		−2.45	*(10)*
6. Galactose-6-phosphate + H_2O ⇅ Galactose + P_i	A	38	8.5	?	2.3	−3.0	*(8)*
7. Glucose-6-phosphate ⇅ Mannose-6-phosphate	A	30	7.4	?	0.30	+0.7	*(18)*
8. Mannose-6-phosphate + H_2O ⇅ Mannose + P_i	A	38	8.5	?	1.40	−2.7	*(8)*
9. Glucose-6-phosphate ⇅ Fructose-6-phosphate	A	30	7.4	?	0.45	+0.5	*(18)*
10. Fructose-6-phosphate + H_2O ⇅	A	38	8.5	?	4.2	−3.4	*(8)*
Fructose + P_i	B	30	7	?	—	−3.8	[From 5C (−3.3) and 9A (−0.5)]
	C	38	5.8	?	8.7	−3.8	*(8)*
11. Fructose-1-phosphate + H_2O ⇅ Fructose + P_i	A	38	8.5	?	1.8	−2.8	*(8)*
	B	38	5.8	?	5.6	−3.5	*(8)*

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[a] Standard free-energy change of reaction at specified temperature and pH; $\Delta F'$ has been calculated from the expression $\Delta F' = -2.30\ RT \log_{10} K_e$; or $-2.30\ RT \log_{10} [K_e \times (H_2O)]$ for hydrolysis.

TABLE I (*Continued*)

Reaction		T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'^a$	References
12. Glucose-1-phosphate + Phosphoglucomutase-phosphate ⇅ Glucose-1,6-diphosphate + Phosphoglucomutase	A	30	7.5	0.001	4.58	−0.9	(*19, 20*)
13. Glucose-1,6-diphosphate + Phosphoglucomutase ⇅ Glucose-6-phosphate + Phosphoglucomutase-phosphate	A	30	7.5	0.001	3.76	−0.8	(*19, 20*)
14. Phosphoglucomutase-phosphate + H_2O ⇅ Phosphoglucomutase + P_i	A	30	7.0	?	—	−4.2	[From 5C (−3.3) and 12A (−0.9)]
						−4.2	[From 16A (−5.0) and 13A (+0.8)]
15. Glucose-1-phosphate ⇅ Glucose-6-phosphate	A	30	7.5	0.008	17	−1.7	(*21*)
	B	25	7.0	0.025	17	−1.7	(*29*)
16. Glucose-1-phosphate + H_2O ⇅ Glucose + P_i	A	30	7.0	0.025	—	−5.0	[From 5C (−3.3) and 15 (−1.7)]

16a. Galactose-1-phosphate + H_2O $\rightleftarrows$ Galactose + P_i	A	38	8.5	?	23–33	−4.4 – −4.6	(*8*)
	B	25	7.0	0.025	—	−5.0	[From 16A, 17aA, and 17bA]
17. ATP + Glucose $\rightleftarrows$ ADP + Glucose-6-phosphate	A	30	6.0	0	386^b(±58)	−3.6	(*22*)
	B	30	6.0	0.08	155^b(±31)	−3.05	(*22*)
	C	30	7.0	0	—	−4.7	(*22*)
	D	30	7.0	0.08	—	−4.4	(*22*)
	E	30	8.0	0	—	−6.1	(*22*)
	F	30	8.0	0.08	—	−5.8	(*22*)
	G	37	7.25	0.005	170^b(±93)	−3.2	(*23, 24*)
17a. Galactose-1-phosphate $\rightleftarrows$ Glucose-1-phosphate	A	37	7.4	0	3	−0.7	(*25, 26*)
17b. Galactose $\rightleftarrows$ Glucose	A	20	—	—	—	−0.8 ± 0.2	(*27, 28*)
17c. ATP + $(\alpha + \beta)$-D-galactose $\rightleftarrows$ ADP + α-D-galactose-1-phosphate	A	25	7.0	0.025	26 ± 3	−1.9 ± 0.1	(*29*)
18. ATP + Glutamate + NH_3 $\rightleftarrows$ ADP + Glutamine + P_i	A	37	6.0	0.01–0.10	400	−3.8	(*30*)
	B	22	7.0	0.01–0.10	1900	−4.7	(*30*)
	C	37	7.0	0.01–0.10	1200	−4.4	(*30*)
	D	37	7.9	0.01–0.10	3000	−4.9	(*30*)

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[b] Extreme value neglected in calculating K_e.

TABLE I (*Continued*)

Reaction		T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'$ [a]	References
19. ATP + H_2O $\rightleftarrows$ ADP + P_i	A	30	7.0	0.01(?)	—	−7.7	[From 17D (−4.4) and 5C (−3.3)]
	B	37	7.25	0.005(?)	—	−6.5	[From 17G (−3.2) and 5C (−3.3)]
	C	37	7.0	?	—	−7.8	[From 18 (−4.4) and glutamine hydrolysis (−3.4) cf. (*31*)]
	D	25	7.0	0.025	—	−7.0	[From 18C (−4.4) and estimated value for glutamine hydrolysis (−2.6), cf. (*32, 33*)
	E	25	7.0	?	—	(i) −6.9 (ii) −7.8	[From hydrolysis of acetyl-CoA (−7.4, i, or −8.3, ii) cf. (*34*) together with 30A (+0.5)]
	F	25	7.5	?	—	−8.9	[From glycolysis and free energy of formation data cf. (*34, 35*)]
	G	approx. 30	approx. 7	—	—	−7.0	[From 20B (−0.4), 31A (+0.6), 26A (−6.7), 27B (−3.1), and 29B (+2.6)]

	H	25	7.0	0.025	—	−6.9	[From 17cA (−1.9) and 16aB (−5.0)]
20. ATP + AMP $\rightleftarrows$ ADP + ADP	A	1	6.0	0	2.9	−0.6	(*36*)
	A′	20	6.0	0	2.4	−0.5	(*36*)
	B	20	6.0	0.001	1.9	−0.4	(*36*)
	C	20	6.0	0.002	1.6	−0.3	(*36*)
	D	25	7.4	0.01	2.2	−0.5	(*37*)
	E	30	7.5	0.01	3.2	−0.7	(*38*)
	F	25	7.0	0.01	2.1	−0.5	(*38a*)
	G	25	7.0	0.025	3.5	−0.7	(*38b*)
21. ADP + H_2O $\rightleftarrows$ AMP + P_i	A	25	7.0	0.01		−6.4	[From 20F (+0.5) and 19H (−6.9)]
22. ATP + H_2O $\rightleftarrows$ AMP + PP_i	A	37	7.5	0.01(?)		−8.6	[From 30A (−0.5), 31A (−0.6), and 19A (−7.5)][c]
23. ITP + ADP $\rightleftarrows$ IDP + ATP	A	37	6.0	0.01	1	0	(*39*)
24. ITP + H_2O $\rightleftarrows$ IDP + P_i	A	25	7.0	0.01(?)		−6.9	[From 19H (−6.9) and 23A (0)]
25. UTP + UMP $\rightleftarrows$ UDP + UDP	A	36	7.5	0.007	0.94	0	(*40*)

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[c] Free-energy change corrected for pH.

TABLE I (*Continued*)

Reaction		T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'$ [a]	References
26. $PP_i + H_2O \rightleftharpoons P_i + P_i$	A	37	6.4	0.02	1000	−6.7	(*41*)
	B	25	7.0	—	—	−8	[Estimated from heats of hydrolysis of PP_i (−5.8) from (*42*); and of ATP (−4.8) from (*32*) and 19A (−7.0)]
	C	30	approx. 7	—	—	−6.5	[From 27B (+3.1), 29B (−2.6), 21A (−6.4),[c] and 31A (−0.6)]
27. Acetyl phosphate + ADP $\rightleftharpoons$ Acetate + ATP	A	37	?	?	177	−3.2	(*43*)
	B	29	7.0	0.01	167	−3.1	(*44*)
	C	?	?	?	77	−2.6	cf. (*45*)
	D	29	7.3	0.01	110	−2.8	(*46*)
28. Acetyl phosphate + $H_2O \rightleftharpoons$ Acetate + P_i	A	29	7.0	—	—	−10.1	[From 19A (−7.0) and 27B (−3.1)]
29. Acetyl phosphate + CoA $\rightleftharpoons$ Acetyl-CoA + P_i	A	28	8.1	?	58	−2.4	(*47*)
	B	28	8	?	74	−2.6	(*48*)

30. Acetate + ATP + CoA ⇅ Acetyl-CoA + ADP + P_i	A	28	7–8	?	—	+0.5	[From 27B (+3.1) and 29B (−2.6)]
31. Acetate + CoA + ATP ⇅ Acetyl-CoA + AMP + PP_i	A	37	7.5	0.01	2.7	−0.6	*(49, 50)*
	B	38	7.5–8.0	0.0005	0.86	+0.1	*(51)*
31a. Propionate + CoA + ATP ⇅ Propionyl-CoA + AMP + PP_i	A	38	8.0	0.0005	1.15	−0.1	*(51)*
32. Heptanoate + CoA + ATP ⇅ Heptanoyl-CoA + AMP + PP_i	A	38	8.0	?	1.11	−0.1	*(52)*
33. Acetylimidazole + P_i ⇅ Acetyl phosphate + imidazole	A	26	6.1–7.3	0	125	−2.8	*(53)*
34. Arginine phosphate + ADP ⇅ Arginine + ATP	A	20–45	6.1	0	500	−3.8	*(54)*
	B	20–45	9.1	0	6	−1.1	*(54)*
	C	20–45	9.1	0.01	12	−1.5	*(54)*
	D	20	7.7	0.001	3.1	−0.7	*(55)*
	E	5	6.9	0.0002	0.76	+0.2	*(56)*
	F	5	7.3	0.0002	0.38	+0.6	*(56)*
	G	5	7.6	0.0002	0.20	+0.9	*(56)*
	H	5	7.8	0.0002	0.11	+1.2	*(56)*
	I	5	6.6	0.001	4.45	−0.9	*(56)*
	J	5	7.1	0.001	1.98	−0.4	*(56)*
	K	5	7.6	0.001	0.76	+0.2	*(56)*
	L	5	7.9	0.001	0.38	+0.6	*(56)*
	M	5	7.6	0.01	4.45	−0.9	*(56)*

Table I (*Continued*)

Reaction		T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'$ [a]	References
	N	5	7.7	0.01	1.98	−0.4	(*56*)
	O	5	8.1	0.01	0.76	+0.2	(*56*)
	P	5	8.3	0.01	0.38	+0.6	(*56*)
35. Arginine phosphate + H_2O ⇅ Arginine + P_i	A	25	6.9	0.0002	—	−7.0	[From 34E (+0.2) and 19A (−7.2)[d]]
	B	25	7.3	0.0002	—	−6.9	[From 34F (+0.6) and 19A (−7.5)][d]
	C	25	7.6	0.0002	—	−6.9	[From 34G (+0.9) and 19A (−7.8)][d]
	D	25	7.6	0.01	—	−8.5	[From 34M (−0.9) and 19A (−7.6)][c]
	E	25	8.1	0.01	—	−8.1	[From 34O (+0.2) and 19A (−8.3)][c]
	F	25	8.3	0.01	—	−8.0	[From 34P (+0.6) and 19A (−8.6)][c]
36. Creatine phosphate + ADP ⇅ Creatine + ATP	A	20	7.7	0.001	7.5	−1.2	(*55*)
	B	20	8.8	0.001	2.1	−0.4	(*55*)
	C	?	8.6	?	25	−1.9	(*57*)
	D	37	10	0.003	26	−2.0	(*58*)
	E	30	8.9	0.001	3.1	−0.7	(*59*)
	F	30	7.4	0.002	83	−2.6	(*59*)
	G	30	8.0	0.002	22	−1.7	(*59*)
	H	30	8.9	0.002	3.6	−0.8	(*59*)
	I	30	9.8	0.002	0.83	+0.1	(*59*)
	J	30	7.4	0.006	102	−2.8	(*59*)
	K	30	8.0	0.006	26	−2.0	(*59*)
	L	30	8.9	0.006	4.8	−0.9	(*59*)

	M	30	9.8	0.006	1	0	*(59)*
	N	30	7.4	0.01	105	−2.8	*(59)*
	O	30	8.0	0.01	28	−2.0	*(59)*
	P	30	8.9	0.01	4.7	−0.9	*(59)*
	Q	30	9.8	0.01	1.1	0	*(59)*
	R	30	7.4	0.02	105	−2.8	*(59)*
	S	30	8.0	0.02	28	−2.0	*(59)*
	T	30	8.9	0.02	5.3	−1.0	*(59)*
	U	30	9.8	0.02	1.2	−0.1	*(59)*
37. Creatine phosphate + H_2O ⇅ Creatine + P_i	A	25	7.4	0.01	—	−10.2	[From 36N (−2.8) and 19A (−7.4)][c]
	B	25	8.0	0.01	—	−10.2	[From 36O (−2.0) and 19A (−8.2)][c]
	C	25	8.9	0.01	—	−10.3	[From 36P (−0.9) and 19A (−9.4)][c]
	D	25	9.8	0.01	—	−10.6	[From 36Q (0) and 19A (−10.6)][c]
38. Creatine phosphate + RNA ⇅ Creatine + phosphorylated RNA	A	37	10	0.003	100	−2.8[e]	*(58)*
39. Phosphorylated RNA + H_2O ⇅ RNA + P_i	A	37	10	0.003	—	−10.3[e]	[From 38A (+2.8) and 36D (−2.0) and 19A (−11.1)][d]
40. Coenzyme A-S-phosphate + ADP ⇅ Coenzyme A + ATP	A	?	approx. 7	?	29	−2.0	*(60)*
	B	37	7.2–7.4	?	0.7	+0.2	*(61)*

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[d] Free-energy change corrected for pH and Mg^{2+} concentration.

[e] Estimated value if all reactive phosphoryl groups are equivalent.

Table I (*Continued*)

Reaction		T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'$ [a]	References
41. Coenzyme A-S-phosphate + H_2O ⇵	A	25	7	?	—	−9	[From 40A (−2.0) and 19A (−7.0)]
Coenzyme A + P_i	B	37	7.3	?	—	−7.1	[From 40B (+0.2) and 19A (−7.3)]
42. β-L-Aspartyl phosphate + ADP ⇵ Aspartate + ATP	A	15	8.0	0.02	2850	−4.5	*(62)*
43. β-L-Aspartyl phosphate + H_2O ⇵ Aspartate + P_i	A	15	8.0	0.02	—	−12.7	[From 42A (−4.5) and 19A (−8.2)][c]
44. 3-Phosphoglyceroyl phosphate	A	25	6.9	?	3200	−4.8	*(63)*
+ ADP ⇵	B	25	6.9	0.005	3200	−4.8	*(64)*
Glycerate-3-phosphate + ATP	C	25	6.9	0	3100	−4.8	*(9, 63)*
45. 3-Phosphoglyceroyl phosphate + H_2O ⇵ Glycerate-3-phosphate + P_i	A	25	6.9	0.005	—	−11.8	[From 44B (−4.8) and 19A (−7.0)]
46. Enolpyruvate phosphate +	A	30	7.4	0.002	6450	−5.3	*(65)*[f]
ADP ⇵	B	30	8.0	0.002	2210	−4.7	*(65)*[f]
Pyruvate + ATP	C	30	8.4	0.002	914	−4.1	*(65)*[f]
	D	30	approx. 7.6	0.003	2100	−4.6	*(14)*

47. Enolpyruvate-2-phosphate^{3-} + IDP^{3-} + HCO_3^- + H^+ $\rightleftarrows$ Oxaloacetate^{2-} + ITP^{4-} + H_2O	A	30	7.6	0.002	5.86 × 10^6	−9.3, ($\Delta F°$)	*(66)*
48. Enolpyruvate-2-phosphate + H_2O $\rightleftarrows$ Pyruvate + P_i	A	30	7.4	—	—	−12.7	[From 46A (−5.3) and 19A (−7.4)][c]
	B	30	7.6	—	—	−12.2	[From 46D (−4.6) and 19A (−7.6)][c]
	C	30	8.0	—	—	−12.9	[From 46B (−4.7) and 19A (−8.2)][c]
	D	30	8.4	—	—	−12.9	[From 46C (−4.1) and 19A (−8.8)][c]
	E	30	7.6	—	—	−13.0	[From 47A (−9.3), 24A (−7.6),[c] and thermal data, cf. *(34)*
49. Maltose + P_i $\rightleftarrows$ Glucose + Glucose-1-phosphate	A	37	7.0	?	0.23	+0.5	*(67)*
50. Sucrose + P_i $\rightleftarrows$ Glucose-1-phosphate + Fructose	A	30	6.6	?	20	−1.8	*(68)*
51. $(Hexose)_{n+1}$ + P_i $\rightleftarrows$ $(Hexose)_n$ + Glucose-1-phosphate	A	—	5.0	?	0.09	+1.5	*(69, 70)*
	B	15–30	6.0	?	0.14	+1.2	*(71, 72)*
	C	15–30	7.0	?	0.33	+0.7	*(71, 72)*
	D		7.0	?	0.32	+0.7	*(69, 70)*

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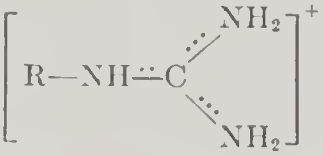
[f] For a recent extension of this work, see reference *65a*.

TABLE I (*Continued*)

Reaction	T (°C)	pH	Mg^{2+} (Molar)	K_e	$\Delta F'$ [a]	References
52. Nicotinamide riboside + P_i $\rightleftarrows$ Nicotinamide + Ribose-1-phosphate + H^+	A 25	7	?	10^3	−4.2, ($\Delta F°$)	(*73, 74*)
	B 25	8	?	10^4	−5.6, ($\Delta F°$)	(*74*)
52a. Deoxyinosine + P_i $\rightleftarrows$ Hypoxanthine + Deoxyribose-1-phosphate	A 24	7.4	?	0.02	+2.3	(*75*)
53. DPN + PP_i $\rightleftarrows$ NMN + ATP	A 38	7.4	0.003	2	−0.4	(*76*)
54. Orotidine-5-phosphate + PP_i $\rightleftarrows$ Orotate + PRPP	A approx. 25	8.0	0.002	8.3	−1.2	(*77*)
55. Aminoimidazolecarboxamide ribotide + PP_i $\rightleftarrows$ Aminoimidazolecarboxamide + PRPP	A 38	8.0	0.002	0.1	+1.4	(*78*)
56. AMP + PP_i $\rightleftarrows$ Adenine + PRPP	A 36	8.0	0.002	1.4×10^{-5}	+6.8	(*79*)

This prediction is supported by the measurements of Noda and coworkers (*59*). As mentioned above, these workers determined the equilibrium of the creatine kinase reaction for a number of concentrations of H^+, Mg^{2+}, ADP, ATP, creatine, and creatine phosphate. When [(ADP) + (ATP)] was 0.001 *M* and [(creatine) + (creatine phosphate)] was 0.032 *M*—conditions which approximate to those in skeletal muscle (*104*)—the influence of Mg^{2+} concentration was not pronounced. From the equilibrium data the free energy of phosphorylation of ADP by creatine phosphate has been calculated. If the free energy of hydrolysis of ATP in this pH range is derived as indicated above, the free energies of hydrolysis of creatine phosphate can also be calculated for different pH values (cf. Table I). These $\Delta F'$ values are consistent with those calculated from the results of Lehmann (*55*), Banga (*57*), and Bresler *et al.* (*58*). However, there are uncertainities about the hydrogen and magnesium ion concentrations in the enzymatic systems used by these workers.

Dixon (*84*), Hill and Morales (*97*), and Kaplan (*86*) have discussed the increase in phosphoryl transfer potential of creatine phosphate at lower pH values. The hydrolysis products, creatine cation



and inorganic phosphate, have a greater resonance energy than creatine phosphate.

b. *Arginine phosphate.* Whereas creatine phosphate is widely distributed in both vertebrate and invertebrate tissues, arginine phosphate is confined to invertebrates. Just as the free energy of hydrolysis of creatine phosphate was calculated above, that of arginine phosphate may be obtained from the reaction catalyzed by arginine kinase,

$$\text{Arginine}^{+}\text{-phosphate}^{2-} + \text{ADP}^{3-} + \text{H}^{+} \rightleftarrows \text{Arginine}^{+} + \text{ATP}^{4-}$$

Griffith *et al.* (*56*) have studied the equilibrium of this reaction between pH 6.5 and pH 8.3, and at a number of concentrations of Mg^{2+}. From their results, free energies of phosphoryl transfer from arginine phosphate to ADP and to water have been calculated. Representative values are shown in Table I. It is seen that for any one Mg^{2+} concentration there is no significant variation of the free energy of hydrolysis in this pH range. It is interesting to note, however, that the free energy of phosphorylation of ADP by arginine phosphate decreases with decreasing Mg^{2+} concentration.

From Table I, it is clear that, under similar conditions, the phosphoryl transfer potential of creatine phosphate is greater than that of arginine phosphate. It is probable that this is partly due to a greater increase of resonance energy on hydrolysis of the former compound. In addition, the presence of a net positive charge on arginine must decrease the repulsion between the hydrolysis products, whereas this effect is absent in the case of creatine phosphate.

5. *Acyl Phosphates*

Phosphate groups bound by an anhydride link to a carboxyl group have particularly high transfer potentials. Acetyl phosphate ($\Delta F'$ of hydrolysis, —10.1 kcal./mole) has a smaller potential than either 3-phosphoglyceroyl phosphate ($\Delta F'$ of hydrolysis, —11.8 kcal./mole) or β-L-aspartyl phosphate ($\Delta F'$ of hydrolysis, —11.5 kcal./mole). The carboxylate and inorganic phosphate formed on hydrolysis have greater resonance energy than the phosphoanhydrides. Ionic repulsion must also be considered, as in the case of pyrophosphate, and probably contributes less to the transfer potential of acetyl phosphate than to that of either of the other acyl phosphates.

The influence of this type of ionic repulsion is seen in the cases of glycerate-2-phosphate and the isomeric glycerate-3-phosphate (see Table I). The free-energy change for the reaction

$$\text{Glycerate-2-phosphate} \rightleftarrows \text{Glycerate-3-phosphate}$$

is —1.1 kcal./mole. Closer proximity of the phosphate to the carboxylate group results in greater ionic repulsion, and accounts for the greater transfer potential of glycerate-2-phosphate. Phytate (inositol hexaphosphate) should be an extreme example of this effect.

6. *Enolpyruvate Phosphate*

This compound has the highest known phosphoryl transfer potential for any metabolite. From McQuate's (*65*) measurements of the equilibrium constant of the reaction

$$\text{Enolpyruvate phosphate} + \text{ADP} \rightleftarrows \text{Pyruvate} + \text{ATP}$$

it has been calculated (see Table I) that the free energy of hydrolysis (—12.8 kcal./mole) is independent of H^+ concentration between pH 7.4 and pH 8.4, as predicted by Dixon (*84*).

After hydrolysis of enolpyruvate phosphate to enolpyruvate and inorganic phosphate the enol isomerizes almost entirely to the stable keto form, pyruvate. The very large free energy of isomerization is probably the main component of the apparent free energy of hydrolysis.

7. *Coenzyme A Phosphate*

This compound was recently identified as an important metabolic intermediate (*60, 61, 105, 106*). From the equilibrium of the CoA thiolkinase reaction,

$$\text{Coenzyme A—S—P} + \text{ADP} \rightleftarrows \text{Coenzyme A—SH} + \text{ATP},$$

Smith *et al.* (*60*) found that the ΔF of phosphate transfer was about -2 kcal./mole. Thus the $\Delta F'$ of hydrolysis of coenzyme A phosphate is about -9.0 kcal./mole, assuming that the $\Delta F'$ of hydrolysis of ATP is -6.9 kcal./mole at pH 7.

However, the earlier report by Feuer and Wollemann (*61*) indicates that the $\Delta F'$ of hydrolysis of coenzyme A phosphate is only -6.8 kcal./mole (see Table I). Whichever value is correct, it is clear that coenzyme A phosphate, like acetyl coenzyme A, has a high group transfer potential.

8. *Phosphorylated Enzymes*

It has been suggested for a number of enzymatic reactions that a phosphorylated enzyme acts as an essential intermediate. The free energy of hydrolysis of the phosphate in one such intermediate has now been measured. Sidbury and Najjar (*19, 20*) have measured the free-energy changes for the successive reactions catalyzed by phosphoglucomutase,

$$\text{Glucose-1-phosphate} + \text{Enzyme-phosphate} \rightleftarrows \text{Glucose-1,6-diphosphate} + \text{Enzyme} \quad (\Delta F',\ -0.9 \text{ kcal./mole phosphate})$$

$$\text{Glucose-1,6-diphosphate} + \text{Enzyme} \rightleftarrows \text{Glucose-6-phosphate} + \text{Enzyme-phosphate} \quad (\Delta F',\ -0.8 \text{ kcal./mole phosphate})$$

For the over-all reaction

$$\text{Glucose-1-phosphate} \rightleftarrows \text{Glucose-6-phosphate}$$

the free-energy change is -1.7 kcal./mole. Since the free energy of hydrolysis of glucose-6-phosphate is -3.3 kcal./mole (cf. Table I), that of glucose-1-phosphate is -5.0 kcal./mole. If the free energy of hydrolysis of the glycosyl phosphates of glucose-1-phosphate and glucose-1,6-diphosphate are equal, the free energy of hydrolysis of the phosphorylated enzyme is -4.2 kcal./mole of enzyme phosphate. As pointed out by Sidbury and Najjar (*19, 20*), this low value makes it most unlikely that the phosphorylated residue is an enol, acyl, or guanidine phosphate. This value would be consistent with that expected for a phosphorylated tyrosine residue (cf. Table II).

9. *Other Phosphates*

Carbamyl phosphate (*107*) and γ-glutamyl phosphate (cf. *89*) both participate in important metabolic reactions, from which it appears that they have high phosphoryl transfer potentials. Equilibrium values for the reactions in which these are involved have not yet been reported. However, they are structurally analagous to other compounds considered above, which are known to have large $-\Delta F'$ of hydrolysis.

TABLE II
PHOSPHORYL TRANSFER POTENTIALS[a]

Phosphoryl donor	Potential	Phosphoryl donor	Potential
Enolpyruvate phosphate	12.8[b]	ADP	6.4[b]
3-Phosphoglyceroyl phosphate	11.8[b,c]	Substituted phenyl phosphate	3.5 to 5.5[g]
β-L-Aspartyl phosphate	11.5[b]	Galactose-1-phosphate	5.0
Creatine phosphate	10.5[b]	Glucose-1-phosphate	5.0
Acetyl phosphate	10.1[b]	Glycerate-2-phosphate	4.2
Coenzyme A-S-phosphate	9.0[b,d] (or 6.8[b,e])	Phosphoglucomutase phosphate	4.2
Arginine phosphate	9.0[b]	Fructose-6-phosphate	3.8
ATP (α-phosphoanhydride)	8.0[b,f]	Glucose-6-phosphate	3.3
ATP (β-phosphoanhydride)	6.9 ± 0.2	Glycerate-3-phosphate	3.1
ITP (β-phosphoanhydride)	6.9[b]	Fructose-1-phosphate	3.1
Inorganic pyrophosphate	6.6	Glycerol-1-phosphate	2.3

[a] Phosphoryl transfer potentials are expressed as $-\Delta F'$ in kcal./mole at pH 7.0 and 25° in the presence of about 0.01 *M* Mg^{2+}. These values have been derived from the information given in Table I. Errors arising in calculation for this pH and Mg^{2+} concentration are probably less than 0.4 kcal./mole.

[b] These values are subject to the same errors as the potential of ATP.

[c] Potential of acyl phosphate only.

[d] Smith *et al.*, 1957 (*60*).

[e] Feuer and Wollemann, 1956 (*61*).

[f] Transfer potential of pyrophosphoryl group.

[g] Estimated from heats and entropies of hydrolysis (cf. ref. *15*).

As seen from Table II, the transfer potentials of phosphorylated metabolites may be arranged in a continuous series from glycerol phosphate (2.3 kcal.) to enolpyruvate phosphate (12.8 kcal.). This shows that the arbitrary division of phosphorylated metabolites into "high-energy" and "low-energy" groups is no longer justified. However, Lipmann's (*81*) suggestion that transfer potential largely determined metabolic reactivity of phosphorylated compounds has undoubtedly guided the development of knowledge of intermediary metabolism.

D. FREE ENERGIES OF PHOSPHOROLYSIS AND PYROPHOSPHOROLYSIS

There are many metabolic reactions in which interaction with inorganic phosphate or pyrophosphate, rather than with water, leads to bond cleavage, as shown (Reactions 1–3).

$$A\text{—}B + H_2O \rightleftarrows A\text{—}H + B\text{—}OH \quad \text{(i.e., hydrolysis)} \tag{1}$$

$$A\text{—}B + HO\text{—}PO_3H_2 \rightleftarrows A\text{—}H + B\text{—}O\text{—}PO_3H_2 \quad \text{(i.e., phosphorolysis)} \tag{2}$$

$$A\text{—}B + HO\text{—}PO(OH)OPO_3H_2 \rightleftarrows A\text{—}H + B\text{—}O\text{—}PO(OH)OPO_3H_2 \quad \text{(i.e., pyrophosphorolysis)} \tag{3}$$

Since in Reactions 2 and 3 the phosphates (B—OPO_3H_2) and pyrophosphates (B—$OPO(OH)OPO_3H_2$) formed have negative free energies of hydrolysis, the free energy changes of these reactions must be less negative than that of the corresponding hydrolysis (Reaction 1). Consequently, the equilibria established by phosphorolysis and pyrophosphorolysis may frequently be determined in cases where the equilibria of the corresponding hydrolytic reactions cannot be conveniently measured.

Just as free energies of hydrolysis of phosphates are a useful indication of their tendency to transfer phosphoryl groups, so the free energy of phosphorolysis or pyrophosphorolysis of a series of analogous compounds may be used to compare their group transfer potentials.

Transfer of glucosyl groups from sucrose and from glycogen to inorganic phosphate have been studied:

$$\text{Sucrose} + P_i \rightleftarrows \text{Fructose} + \text{Glucose-1-phosphate} \quad (\Delta F',\ -1.8\ \text{kcal./mole})^*$$

$$\underset{\text{(i.e., glycogen)}}{(\text{Glucose})_n} + P_i \rightleftarrows (\text{Glucose})_{n-1} + \text{Glucose-1-phosphate} \quad (\Delta F',\ +0.6\ \text{kcal./mole})^*$$

From these free energies of phosphorolysis it can be seen that the glucosyl transfer potential of sucrose is 2.4 kcal./mole greater than that of glycogen (cf. *108*).

The increasing number of known phosphorolytic and pyrophosphorolytic reactions (see Tables VII and VIII) will undoubtedly increase the value of this aspect of the thermodynamics of phosphate transfer.

E. COUPLING OF EXERGONIC AND ENDERGONIC REACTIONS

Reactions with negative free energies have been termed exergonic and those with positive free energies, endergonic. Formation of products in the second type of reaction is not thermodynamically favorable, the equilibrium constant being less than unity. Almost all the reactions characteristic of growth (e.g., protein, polysaccharide, and nucleic acid synthesis) formally involve the joining together of small units (amino acids, sugars, and nucleotides) with elimination of water, to form products of higher molecular weight, as for example

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* See Table I.

$$\begin{aligned}&\text{R—NH—CHR}'\text{—CO—OH} + \text{H—NH—CHR}''\text{—CO—NH—R}'''\\ &\qquad\rightarrow \text{R—NH—CHR}'\text{—CO—NH—CHR}''\text{—CO—NH—R}''' + H_2O\end{aligned}$$

Such reactions have to take place at great dilutions in an aqueous environment and, as discussed in a previous section, reversal of hydrolysis in these circumstances is thermodynamically unfavorable.

In living things such endergonic reactions, which would not result in accumulation of materials required during growth, are of little metabolic importance. Instead, one or more of the reactants is activated by group transfer (e.g., of a phosphoryl, pyrophosphoryl, or adenyl group) from a reactive compound (e.g., ATP). Provided this reactive compound can be constantly regenerated (e.g., by oxidative phosphorylation) net synthesis of protein, polysaccharide, or nucleic acid is an exergonic reaction which, as far as thermodynamic factors are involved, may proceed continuously. Some enzymatic processes of this sort are illustrated by the following examples.

1. *Glycogen Synthesis*

If glycogen synthesis involved the direct condensation of glucose (9),

$$(\text{Hexose})_n + \text{Glucose} \rightleftarrows (\text{Hexose})_{n+1} + H_2O \qquad (\Delta F',\ +3.7\ \text{kcal./mole})$$

the equilibrium would be most unfavorable. The individual reactions which may take place in glycogen biosynthesis,

$$\text{ATP} + \text{Glucose} \rightleftarrows \text{Glucose-6-phosphate} + \text{ADP} \qquad (\Delta F',\ -3.6\ \text{kcal./mole})^*$$

$$\text{Glucose-6-phosphate} \rightleftarrows \text{Glucose-1-phosphate} \qquad (\Delta F',\ +1.7\ \text{kcal./mole})$$

$$(\text{Hexose})_n + \text{Glucose-1-phosphate} \rightleftarrows (\text{Hexose})_{n+1} + P_i \qquad (\Delta F',\ -0.6\ \text{kcal./mole})$$

result in the over-all reaction

$$(\text{Hexose})_n + \text{Glucose} + \text{ATP} \rightleftarrows (\text{Hexose})_{n+1} + \text{ADP} + P_i \qquad (\Delta F',\ -2.5\ \text{kcal./mole})$$

---

[*] See Table I.

This reaction must proceed in the direction of glycogen synthesis if ADP is continuously phosphorylated to ATP. An alternative mechanism is discussed in references *108a,b,c.*

2. *Activation of Acetate*

The active acetyl donor, acetyl coenzyme A, is formed from coenzyme A and acetate by alternative reaction sequences.* The reaction is thermodynamically unfavorable:

$$\text{Acetate} + \text{CoA} \rightleftarrows \text{Acetyl-CoA} + H_2O \qquad (\Delta F', +7.5 \text{ kcal./mole})^*$$

In animal tissues and yeast this activation is brought about by the successive reactions

$$\text{Acetate} + \text{ATP} \rightleftarrows \text{Acetyl adenylate} + PP_i$$

and

$$\text{Acetyl adenylate} + \text{CoA} \rightleftarrows \text{Acetyl-CoA} + \text{AMP}$$

The free-energy change of the over-all reaction

$$\text{Acetate} + \text{CoA} + \text{ATP} \rightleftarrows \text{Acetyl-CoA} + \text{AMP} + PP_i$$

is —0.6 kcal./mole.*

In bacterial systems the activation of acetate is achieved by the following reactions:

$$\text{Acetate} + \text{ATP} \rightleftarrows \text{Acetyl phosphate} + \text{ADP} \qquad (\Delta F', +3.1 \text{ kcal./mole})^*$$

and

$$\text{Acetyl phosphate} + \text{CoA} \rightleftarrows \text{Acetyl-CoA} + P_i \qquad (\Delta F', -2.6 \text{ kcal./mole})^*$$

$\Delta F'$ of the over-all reaction

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftarrows \text{Acetyl-CoA} + \text{ADP} + P_i$$

is +0.5 kcal./mole.

In both these cases, the equilibrium constant of the net reaction is close to unity, and the continuous supply of ATP by oxidative reactions ensures that the over-all reactions proceed in the direction of synthesis of acetyl-CoA.

3. *Glutamine Synthesis*

Though glutamine synthesis by the reaction

$$\text{Glutamate} + NH_3 \rightleftarrows \text{Glutamine} + H_2O \qquad (\Delta F', \text{about } 3.5 \text{ kcal./mole})\ (31)$$

---

* See Table I.

is thermodynamically unfavorable, it takes place readily in the reaction catalyzed by glutamine synthetase:

$$\text{Glutamate} + \mathrm{NH_3} + \text{ATP} \rightleftarrows \text{Glutamine} + \text{ADP} + \mathrm{P}_i \qquad (\Delta F', -4.4 \text{ kcal./mole})^*$$

This reaction probably represents the sum of the successive steps (*89*)

$$\text{Glutamate} + \text{ATP} \rightleftarrows \text{Glutamyl phosphate} + \text{ADP}$$

and

$$\text{Glutamyl phosphate} + \mathrm{NH_3} \rightleftarrows \text{Glutamine} + \mathrm{P}_i$$

Formation of the amide bond in glutamine synthesis is analogous to the formation of peptide bonds during protein synthesis. The synthesis of protein from amino acids is also an endergonic reaction ($\Delta F'$, + 2 to + 4 kcal./mole of peptide) (*109*).† This is promoted by activation of the amino acids in reactions which ultimately depend on a supply of ATP. Condensation of the activated amino acids is a thermodynamically favorable reaction.

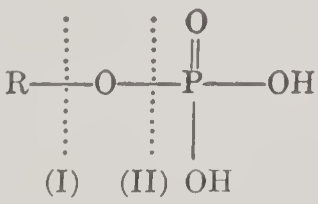
From consideration of reactions such as those discussed above, it is clear that ATP has a predominant role in the activation of metabolites. However, Table II shows that it is not thermodynamically unique. The almost universal participation of ATP in such reactions must be attributed to structural features which result in lower activation energies of enzymatic group transfer from this nucleotide than from analogous nucleoside triphosphates.

III. Enzymatic Synthesis of Phosphates

A. PHOSPHORYL AND PHOSPHATE TRANSFER REACTIONS

From free energy data of the type already considered it is possible to predict the composition of many equilibrium mixtures. Favorable free-energy changes, however, do not ensure that reactions will take place at a measurable rate. Almost all reactions in the living cell take place at useful rates because they are catalyzed by enzymes. The following sections, therefore, describe the reactions involved in the biosynthesis of phosphates.

Phosphates, with the general formula shown



$$\mathrm{R{-}O{-}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}{-}OH}$$

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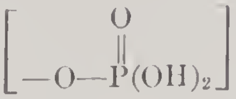
* See Table I.

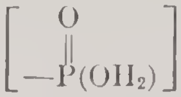
† For a recent discussion see Lipmann (*110*) and following papers.

may be cleaved at I or II. Cleavage at I results in transfer of the phosphate group



$$\left[\text{—O—}\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}(\mathrm{OH})_2\right]$$

in which oxygen and phosphorus atoms are transferred together. When cleavage occurs at II, the oxygen atom is not transferred with the phosphorus; this cleavage results in transfer of the phosphoryl group.

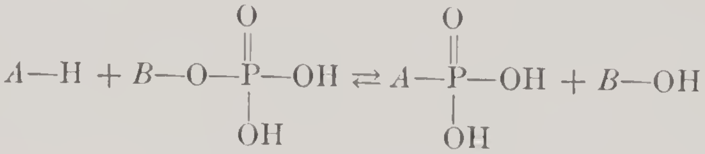


$$\left[\text{—}\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}(\mathrm{OH_2})\right]$$

These processes are most readily recognized by the use of compounds containing O^{18} and P^{32}. This is well illustrated by the pioneer work of Cohn (*111*), who found that the nonenzymatic hydrolysis of glucose-6-phosphate by acid involved cleavage at I, whereas acid phosphatase at a similar pH caused cleavage at II. Under alkaline conditions, both enzymatic and nonenzymatic hydrolysis remove a phosphoryl group (cleavage at II).

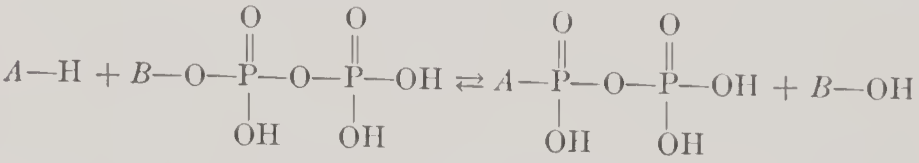
The following types of enzymatic reactions are involved in biosynthesis of phosphates:

a. Transfer of a phosphoryl or pyrophosphoryl group (phosphorylation and pyrophosphorylation):



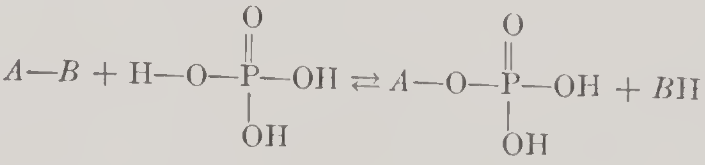
$$A\text{—H} + B\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} \rightleftarrows A\text{—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} + B\text{—OH}$$

and



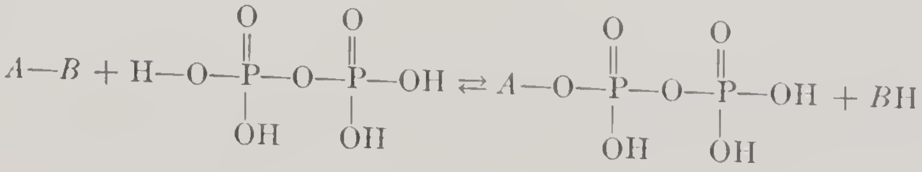
$$A\text{—H} + B\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} \rightleftarrows A\text{—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} + B\text{—OH}$$

b. Bond cleavage by inorganic phosphate or pyrophosphate (phosphorolysis and pyrophosphorolysis):



$$A\text{—}B + \text{H—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} \rightleftarrows A\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} + B\mathrm{H}$$

and

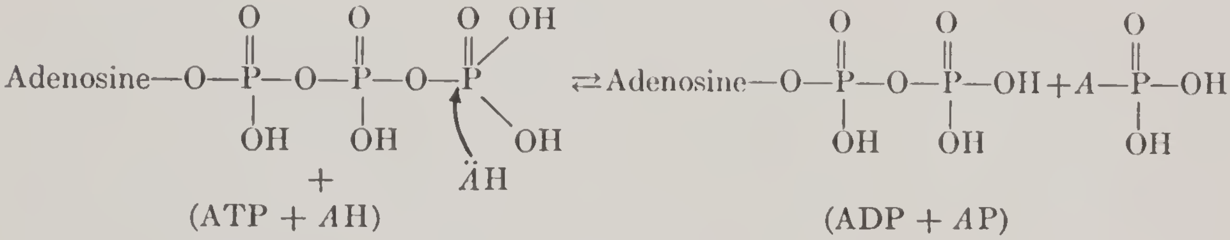


$$A\text{—}B + \text{H—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} \rightleftarrows A\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—O—}\underset{\substack{|\\ \mathrm{OH}}}{\overset{\substack{\mathrm{O}\\ \|}}{\mathrm{P}}}\text{—OH} + B\mathrm{H}$$

Each of these types of reaction, and the enzymes which catalyze them, are discussed in the following sections. Although a rational system has been proposed (*112*) there is no universally-accepted nomenclature for the enzymes involved in these biosyntheses. The names in common use are adopted here.

1. *Phosphoryl Transfer from ATP and Related Nucleotides*

This reaction may be represented as a nucleophilic attack by the phosphoryl acceptor, *A*—H, on the electrophilic terminal phosphorus of ATP:



$$\text{Adenosine—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\underset{|}{\text{P}}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\underset{|}{\text{P}}}}}\text{—O—}\overset{\text{O}\ \ \text{OH}}{\text{P}}\text{—OH} + \ddot{A}\text{H} \rightleftarrows \text{Adenosine—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\underset{|}{\text{P}}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\underset{|}{\text{P}}}}}\text{—OH} + A\text{—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\underset{|}{\text{P}}}}}\text{—OH}$$

(ATP + *A*H) (ADP + *A*P)

Such reactions are catalyzed by enzymes called kinases, (or alternatively phosphotransferases or phosphoferases), and account for the biosynthesis of many of the organic monophosphates found in living tissues. Evidence for phosphoryl transfer has been established with isotopes and is directly apparent for the kinase reactions involving guanidine phosphates.

These enzymes are inactivated by reagents (e.g., iodoacetamide and *p*-chlormercuriphenyl derivatives) which react with free —SH groups. It seems likely from recent studies with a number of kinases that ATP is coordinated with magnesium, which is bound to the enzyme, probably through these —SH groups (*56, 84*).

Kinases are usually rather specific towards both phosphoryl donors and acceptors. In earlier studies, only ATP was found to act as a donor. The increasing availability of nucleoside polyphosphates other than ATP has permitted a re-examination of the specificity of these enzymes towards phosphoryl donors. Both uridine triphosphate and inosine triphosphate function with hexokinase, and act as phosphoryl donors with pyruvic kinase (see Table III). Although such reactions may be due to the presence of trace amounts of ADP and nucleoside diphosphate kinase, this now seems unlikely in the case of pyruvic kinase (*113*). It has also been shown that 3-phosphoglyceroyl phosphate is a phosphoryl donor with this enzyme (*114*).

Kinases will frequently catalyze phosphorylation of several closely related acceptors. Of a large number of nucleosides investigated, only adenosine and 2-aminoadenosine were phosphorylated by adenosine kinase (see Table III). It now appears that deoxy-ADP may be a substrate for pyruvic kinase (*115*). However, there are kinases with surprisingly wide specificities, such as brain hexokinase, which phosphorylates at least 16 compounds, including sorbitan, arabinose, and glucosamine as well as its normal substrate, glucose (*116*).

Compounds which are phosphorylated by ATP and related phosphoryl donors are classified in Table III. The reactions have been grouped according to the nature of the acceptor. Standard free energy changes of these phosphoryl transfers from nucleotides range from about —7 kcal./mole for the reaction with water to +6 kcal./mole for the reaction with pyruvate.

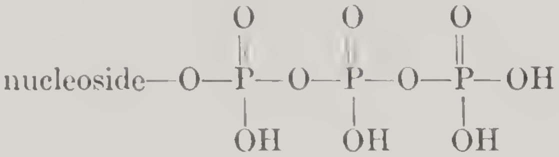
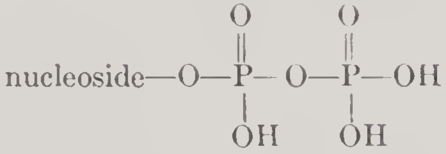
From present knowledge of cell physiology and from consideration of Tables II and III it is clear that kinases belong to three classes. Those of the first class catalyze transfer of phosphoryl groups from compounds of high transfer potential (e.g., 3-phosphoglyceroyl phosphate and enolpyruvate phosphate) to ADP. Equilibria in this class lie far in the direction of ATP formation. In the second class are kinases which transfer phosphoryl groups from ATP to acceptors (e.g., creatine and arginine), thus forming the phosphagens, which have similar transfer potentials to that of ATP and therefore serve as reserves of reactive phosphoryl groups. The free-energy changes of these reactions are small, and are markedly dependent on changes in the H^+ and Mg^{2+} concentration. The third class includes the numerous kinases which phosphorylate, and thus activate, otherwise inert compounds such as glucose and glycerol. In these reactions the equilibria lie far in the direction of phosphorylation of the metabolites, but the initial loss of reactive phosphoryl groups from ATP is more than regained during the subsequent oxidative degradation of the activated metabolites (e.g., glucose-6-phosphate).

There is a metabolic requirement for nucleoside polyphosphates other than those of the adenyl class, particularly as precursors of ribonucleic and deoxyribonucleic acids. These are provided by the action of certain kinases. The adenylate kinase (myokinase) type reaction (Table III, Group X) accounts for the conversion of purine and pyrimidine nucleoside monophosphates, formed from noncyclic precursors, to the diphosphates. These diphosphates are then phosphorylated to the corresponding triphosphates by reaction with ATP (Table III, Group XI). Thus only adenyl nucleotides need be raised to the triphosphate level by oxidative phosphorylation.

2. *Phosphoryl Transfer from Other Phosphates*

Although there are kinases which catalyze the formation of almost all known phosphorylated metabolites the existence of other phosphoryl

TABLE III
PHOSPHORYL TRANSFER FROM ATP AND RELATED COMPOUNDS

Group	Donor	Acceptor	Enzyme[a,b]	Reference
I.	 $\text{nucleoside—O—}\overset{\text{O}}{\underset{\text{OH}}{\text{P}}}\text{—O—}\overset{\text{O}}{\underset{\text{OH}}{\text{P}}}\text{—O—}\overset{\text{O}}{\underset{\text{OH}}{\text{P}}}\text{—OH}$	HOH		
1.	ATP	HOH	Myosin-adenosine triphosphatase[c] (rabbit and frog muscle)	*(117–121)*
2.	ITP		Myosin-adenosine triphosphatase (rabbit and horse muscle)	*(122)*
3.	UTP		Uridine triphosphatase (yeast)	*(123)*
II.	 $\text{nucleoside—O—}\overset{\text{O}}{\underset{\text{OH}}{\text{P}}}\text{—O—}\overset{\text{O}}{\underset{\text{OH}}{\text{P}}}\text{—OH}$	HOH		
1.	GDP	HOH	Nucleoside diphosphatase[c] (pig kidney cortex, yeast)	*(124, 125)*
2.	IDP	HOH	Nucleoside diphosphatase[c] (rat and beef liver, pig kidney cortex, yeast)	*(124–126)*
3.	UDP	HOH	Nucleoside diphosphatase[c] (pig kidney cortex, yeast)	*(124, 125)*
III.	ATP	$R—CH_2—OH$		
1.	ATP	Glycerol (at C-1)	Glycerokinase (liver and kidney of rat and pigeon)	*(127, 128)*
2.	ATP	Glyceraldehyde (at C-3)	Glycerokinase (rat liver)	*(127)*

3.	ATP	Dihydroxyacetone (at C-1)	Glycerokinase (rat liver)	*(127)*
4.	ATP	Glyceric acid (at C-3)	Glyceric kinase (yeast)	*(129)*
5.	ATP	Erythritol	Erythritol kinase (*Propionibacterium pentosaceum*)	*(130)*
6.	ATP	Aminoethanol	Choline kinase (yeast)	*(131)*
7.	ATP	*N*-Methylaminoethanol	Choline kinase (yeast)	*(131)*
8.	ATP	*N*,*N*-Dimethylaminoethanol	Choline kinase (yeast)	*(131)*
9.	ATP	Choline	Choline kinase (yeast)	*(131)*
9a.	ATP	1,5-Sorbitan (at C-6)	Hexokinase (calf brain and heart, rat liver, rat and dog muscle)	*(116, 132)*
9b.	ATP	*myo*-Inositol	Inositol kinase (yeast and rat liver)	*(132a,b,c)*
10.	ATP	2,5-Sorbitan (at C-6)	Hexokinase (brain)	*(116)*
11.	ATP	1,5-Mannitan (at C-6)	Hexokinase (brain)	*(116)*
12.	ATP	Ribose (at C-5)	Pentokinase (yeast)	*(133)*
			Pentokinase (calf liver)	*(134)*

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[a] Only established enzyme names are included; the term "kinase" is used in preference to "phosphokinase."

[b] Wherever possible, the sources of enzymes have been indicated in parentheses. Readily available sources such as yeast and mammalian tissues have been widely studied but the absence of any of these enzymes has rarely been demonstrated in other living systems.

[c] These enzymes may also be regarded as specific phosphatases (cf. Table IV).

TABLE III (*Continued*)

Group (III *Continued*)	Donor	Acceptor	Enzyme[a,b]	Reference
13.	ATP	Deoxyribose (at C-5)	Pentokinase (calf liver)	(*134*)
14.	ATP	Arabinose (at C-5)	Hexokinase (brain)	(*116*)
			Arabinokinase (*Escherichia coli* and yeast)	(*135*)
14a.	ATP	Ribulose (at C-5)	Ribulokinase (*Lactobacillus pentosus*)	(*136*)
15.	ATP	Xylulose (at C-5)	Xylulokinase (*Lactobacillus pentosus* and *Pasteurella pestis*)	(*137, 138*)
16.	ATP	Allose (at C-6)	Hexokinase (brain)	(*116*)
17.	ATP	Altrose (at C-6)	Hexokinase (brain)	(*116*)
18.	ATP	D-Glucose (at C-6)	Hexokinase (yeast)	(*139–141*)
			Hexokinase (animal; cf. 9a)	(*132*)
			Fructokinase (green peas)	(*142*)
19.	ATP	2-Deoxyglucose (at C-6)	Hexokinase (yeast and brain)	(*132, 143*)
20.	ATP	3-Deoxyglucose (at C-6)	Hexokinase (brain)	(*116*)
21.	ATP	D-Glucosamine (at C-6)	Hexokinase (calf brain, yeast, *Neurospora* spp.)	(*116, 144–146*)

22.	ATP	Gluconic acid (at C-6)	Gluconokinase (yeast, *E. coli*)	*(135, 147)*
23.	ATP	2-Ketogluconic acid (at C-6)	2-Ketogluconokinase (*Aerobacter cloacae*)	*(148)*
24.	ATP	Glucosone (at C-6)	Hexokinase (brain)	*(116)*
25.	ATP	D-Mannose (at C-6)	Hexokinase (yeast)	*(140, 141)*
			Fructokinase (green peas)	*(142)*
			Hexokinase (animal; cf. 9a)	*(132)*
26.	ATP	D-Galactose (at C-6)	Hexokinase (brain)	*(116)*
27.	ATP	L-Sorbose (at C-1)	Fructokinase (calf liver and rabbit muscle)	*(149–151)*
28.	ATP	D-Fructose (at C-1)	Fructokinase (calf and rat liver, rabbit muscle)	*(150, 151)*
29.	ATP	D-Fructose (at C-6)	Hexokinase (yeast)	*(141)*
			Hexokinase (brain)	*(116, 152)*
			Fructokinase (plant)	*(142)*
30.	ATP	D-Tagatose (at C-1)	Fructokinase (calf and rat liver, rabbit muscle)	*(150, 151)*
31.	ATP	Glucoheptulose	Hexokinase (brain)	*(116)*
32.	ATP	Sedoheptulose (at C-7)	Heptulokinase (bacteria)	*(153, 154)*
33.	ATP	Mannoheptulose	Hexokinase (brain)	*(116)*

Table III (*Continued*)

Group (III *Continued*)	Donor	Acceptor	Enzyme^{a,b}	Reference
34.	ATP	Ribulose-5-phosphate (at C-1)	Phosphoribulokinase (spinach leaf, yeast)	(*155*)
35.	ATP	Glucose-1-phosphate (at C-6)	Phosphoglucokinase (rabbit muscle, yeast)	(*156, 157*)
36.	ATP	Fructose-1-phosphate (at C-6)	1-Phosphofructokinase (rat liver; rabbit muscle)	(*152*)
37.	ATP	Fructose-6-phosphate (at C-1)	6-Phosphofructokinase (rat liver; rabbit muscle)	(*152, 158, 159*)
38.	ATP	Tagatose-6-phosphate (at C-1)	6-Phosphofructokinase (rabbit muscle)	(*158*)
39.	ATP	Sedoheptulose-7-phosphate (at C-1)	6-Phosphofructokinase (rabbit muscle)	(*158*)
40.	ATP	5-Amino-4-imidazolecarboxamide riboside (at C-5′)	Carboxamide riboside kinase (*E. coli*)	(*160*)
40a.	ATP	Nicotinamide riboside (at C-5′)	Nicotinamide riboside kinase (pig liver)	(*161*)
41.	ATP	Adenosine (at C-5′)	Adenosine kinase (yeast; kidney and liver of rat and rabbit)	(*162–164*)
42.	ATP	2-Aminoadenosine (at C-5′)	Adenosine kinase (yeast)	(*163, 164*)
43.	ATP	Uridine (at C-5′)	Nucleoside kinase (yeast, rat liver)	(*165, 166*)
44.	ATP	Thymidine (at C-5′)	Nucleoside kinase (yeast, *E. coli*)	(*166–168*)
45.	ATP	Deoxyuridine (at C-5′)	Nucleoside kinase (yeast, *E. coli*)	(*166, 168*)
46.	ATP	Pyridoxal	Pyridoxal kinase (yeast, *Streptococcus faecalis* R)	(*169, 170*)

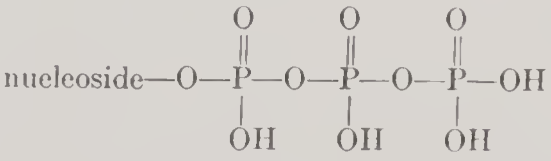
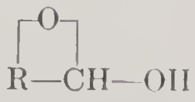
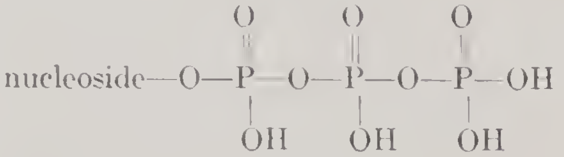
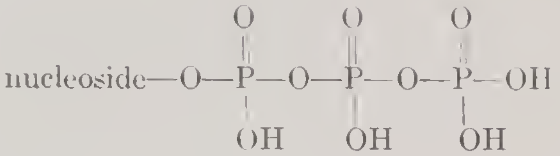
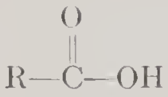
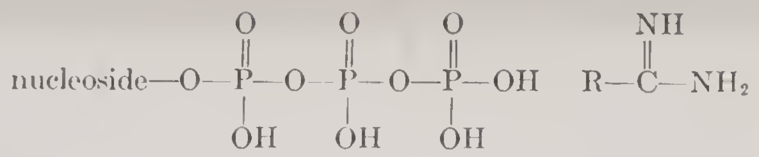
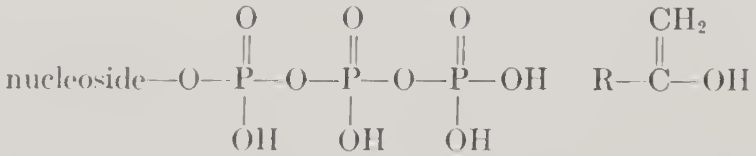
	47. ATP	Riboflavin	Riboflavin kinase (yeast, *Lactobacillus arabinosus*)	(*171, 172*)
	48. ATP	Pantetheine (at ω—OH)	Pantetheine kinase (pigeon liver)	(*173*)
	49. ATP	Dephosphocoenzyme A (at C-3′)	Dephosphocoenzyme A kinase (yeast; rat, pig, and pigeon liver)	(*174, 175*)
	50. ATP	DPN (adenyl ribose at C-2′)	DPN-kinase (yeast; pigeon and rabbit liver, rabbit kidney)	(*174, 176*)
	50a. ATP	Adenylsulfate (at C-3′)	Adenosine phosphosulfate kinase (yeast; rabbit and sheep liver)	(*177–179*)
	51. ATP	Casein (at serine residue)	Casein kinase (rat liver)	(*180*)
	51a. ATP	Liver phosphorylase	Phosphorylase kinase (dog liver)	(*181*)
	51b. ATP	Muscle phosphorylase b	Muscle-phosphorylase b kinase (rat intestinal mucosa)	(*182*)
	52. UTP	Glucose (at C-6)	—	(*161*)
	53. UTP	Fructose-6-phosphate (at C-1)	Phosphofructokinase (rabbit muscle)	(*159*)
	54. ITP	Fructose-6-phosphate (at C-1)	Phosphofructokinase (rabbit muscle)	(*159*)
	55. Phosphorylated RNA	Fructose-6-phosphate (at C-1)	Phosphofructokinase (rabbit muscle)	(*183*)
IV.	 $\text{nucleoside}{-}O{-}\underset{OH}{\overset{O}{\overset{\|}{\underset{\vert}{P}}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\|}{\underset{\vert}{P}}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\|}{\underset{\vert}{P}}}}{-}OH$	 $R{-}\overset{\ulcorner O \urcorner}{CH}{-}OH$ (Hemiacetal)		
	1. ATP	D-Galactose (at C-1)	Galactokinase (yeast, rat liver and brain)	(*26, 184, 185*)
	2. ATP	D-Galactosamine (at C-1)	Galactokinase (galactose-adapted yeast, rat liver and brain)	(*26, 185*)

TABLE III (*Continued*)

Group	Donor	Acceptor	Enzyme^{a,b}	Reference
V.	 $\text{nucleoside}-O-\underset{OH}{\overset{\overset{O}{\Vert}}{P}}-O-\underset{OH}{\overset{\overset{O}{\Vert}}{P}}-O-\underset{OH}{\overset{\overset{O}{\Vert}}{P}}-OH$	RSH		
1.	ATP	Coenzyme A	Coenzyme A kinase	*(60, 61, 105, 106)*
VI.	 $\text{nucleoside}-O-\underset{OH}{\overset{\overset{O}{\Vert}}{P}}-O-\underset{OH}{\overset{\overset{O}{\Vert}}{P}}-O-\underset{OH}{\overset{\overset{O}{\Vert}}{P}}-OH$	 $R-\overset{\overset{O}{\Vert}}{C}-OH$		
1.	ATP	Acetic acid	Acetokinase (*E. coli* and *Streptococcus haemolyticus;* not found in animal tissues)	*(44, 46)*
2.	ATP	Propionic acid	— (*E. coli* and *Streptococcus haemolyticus*)	*(46)*
3.	ATP	3-Phosphoglyceric acid	Phosphoglycerokinase (yeast)	*(63, 64, 129)*
4.	ATP	Succinic acid	Succinate kinase (*Micrococcus lactilyticus*)	*(186)*
5.	ATP	L-Aspartic acid (at β-carboxyl)	Aspartic kinase (yeast)	*(62)*
6.	ATP	Carbonic acid	— (pig heart and rat liver)	*(187, 188)*
7.	ATP	Carbamic acid	— (*Streptococcus faecalis* and rat liver)	*(107)*

VII.	 $\text{nucleoside—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—OH}\quad \text{R—}\overset{\text{NH}}{\overset{\|}{\text{C}}}\text{—NH}_2$			
1.	ATP	Creatine	Creatine kinase[d] (rabbit and sheep muscle)	(*55, 189–193*)
2.	ATP	Arginine	Arginine kinase[d] (crab and crayfish muscle; *Potamobius astacus, P. leptodactylus*)	(*54, 55, 189, 193–195*)
3.	ATP	Canavanine	Arginine kinase[d] (crayfish muscle)	(*195*)
4.	ATP	Homoarginine	Arginine kinase[d] (crayfish muscle)	(*195*)
5.	ATP	Taurocyamine	Taurocyamine kinase[d] (*Arenicola assimilis* muscle)	(*193, 196, 197*)
6.	ATP	β-Guanidinopropionic acid	Taurocyamine kinase[d] (*Arenicola assimilis* muscle)	(*198*)
7.	ATP	Glycocyamine	Glycocyamine kinase[d] (*Arenicola assimilis* muscle)	(*193, 196, 198*)
8.	ATP	Lombricine (β-Guanidinoethyl phosphoryl serine)	— (earthworm)	(*193, 199*)
9.	Phosphorylated RNA	Creatine	Creatine kinase[d] (rabbit muscle)	(*58*)
10.	3-Phosphoglyceroyl phosphate	Creatine	Creatine kinase[d] (rabbit muscle)	(*114*)
VIII.	 $\text{nucleoside—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—OH}\quad \text{R—}\overset{\text{CH}_2}{\overset{\|}{\text{C}}}\text{—OH}$			
1.	ATP	Pyruvic acid	Pyruvic kinase (yeast; rabbit, rat, and human muscle)	(*113, 200, 201*)

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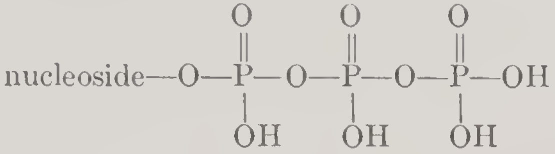
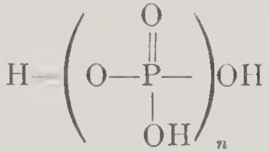
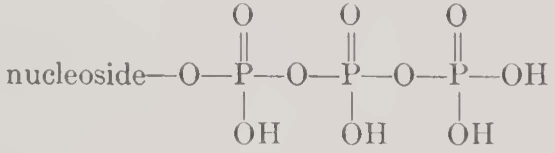
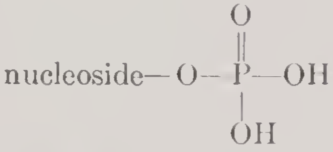
[d] For details of the comparative distribution of phosphagen kinases, see reference *201a*.

TABLE III (*Continued*)

Group (VIII *Continued*)	Donor	Acceptor	Enzyme[a,b]	Reference
2.	GTP	Pyruvic acid	Pyruvic kinase (rabbit muscle)	(*113*)
3.	ITP	Pyruvic acid	Pyruvic kinase (rabbit muscle)	(*113, 161*)
4.	CTP	Pyruvic acid	Pyruvic kinase (rabbit muscle)	(*113*)
5.	UTP	Pyruvic acid	Pyruvic kinase (rabbit muscle)	(*113, 161*)
6.	Deoxy-ATP	Pyruvic acid	Pyruvic kinase	(*115*)
IX.	 $\text{nucleoside}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}OH$	 $H{-}\left(O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}\right)_n OH$		
1.	ATP	Metaphosphate	— (yeast, *E. coli*, and *Aspergillus niger*)	(*89, 202–205; cf. 206*)
X.*	 $\text{nucleoside}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}OH$	 $\text{nucleoside}{-}O{-}\underset{OH}{\overset{O}{\overset{\Vert}{P}}}{-}OH$		
1.	ATP	AMP (at phosphate)	Adenylate kinase (myokinase)	(*207–211*)
2.	ATP	GMP (at phosphate)	—	(*40, 212*)
3.	ATP	UMP (at phosphate)	—	(*213–217*)
4.	ATP	CMP (at phosphate)	—	(*214, 218*)
5.	ATP	Deoxy-AMP[e]	—	(*40, 166, 219–222*)

6.	ATP	Deoxy-GMP[e]	—	*(166, 220, 222)*
7.	ATP	Deoxy-CMP[e]	—	*(166, 218, 221, 222)*
8.	ATP	TMP[e]	—	*(166, 218, 221–223)*
8a.	ATP	RNA[f]	Myokinase (rabbit muscle and guinea pig liver)	*(183, 206, 224, 225*; but cf. *38a)*
9.	GTP	AMP	— (pig kidney)	*(212)*
10.	GTP	GMP		*(40)*
11.	ITP	AMP	— (yeast, calf liver, pig kidney, and pigeon breast muscle)	*(212, 226)*
12.	ITP	Deoxy-AMP	— (*Azotobacter* spp.)	*(221)*
13.	ITP	Deoxy-CMP	— (*Azotobacter* spp.)	*(221)*
14.	CTP	AMP	— (calf liver)	*(214)*

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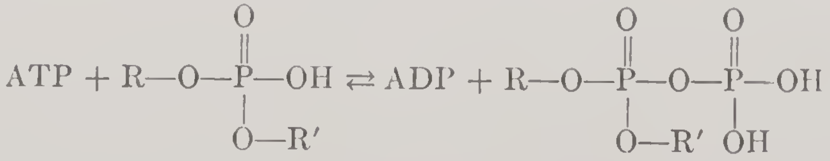
* Note: Reactions in Group X of this table other than 8a, occur widely in animal tissues, plants, yeast, and bacteria. They have been demonstrated particularly in yeast and liver. For a recent discussion of these enzymes, see reference *226a*.

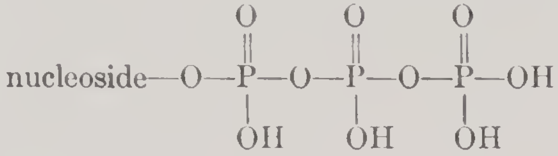
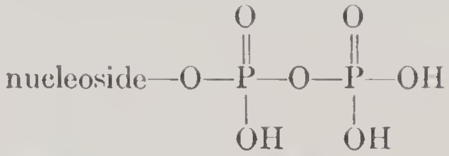
[e] Nucleoside diphosphate is a probable intermediate in conversion of nucleoside monophosphate into nucleoside triphosphate; phosphorylation is at the 5′-phosphate.

[f] Probably:



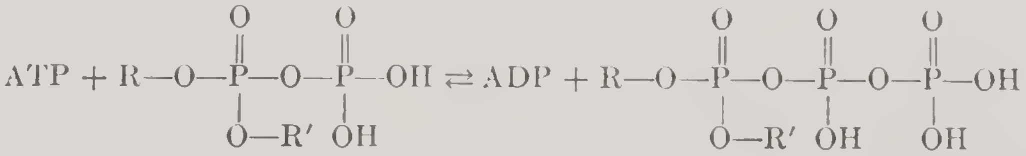
$$\mathrm{ATP} + \mathrm{R{-}O{-}}\underset{\mathrm{O{-}R'}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}OH} \rightleftarrows \mathrm{ADP} + \mathrm{R{-}O{-}}\underset{\mathrm{O{-}R'}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}O{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}OH}$$

TABLE III (*Continued*)

Group (X *Continued*)	Donor	Acceptor	Enzyme[a,b]	Reference
15.	UTP	AMP	— (yeast, calf liver)	(*40, 213–215*)
16.	UTP	UMP	—	(*40, 213, 215*)
XI.	 $\text{nucleoside—O—}\overset{O}{\underset{OH}{P}}\text{—O—}\overset{O}{\underset{OH}{P}}\text{—O—}\overset{O}{\underset{OH}{P}}\text{—OH}$	 $\text{nucleoside—O—}\overset{O}{\underset{OH}{P}}\text{—O—}\overset{O}{\underset{OH}{P}}\text{—OH}$ (at terminal phosphate)		
1.	ATP	GDP	Nucleoside diphosphokinase (pig kidney)	(*212, 227*)
1a.	ATP	IDP	— (yeast, pig kidney, and pigeon breast muscle)	(*39, 226, 227*)
2.	ATP	CDP	— (rat liver)	(*218*)
3.	ATP	UDP	— (yeast; pig kidney)	(*39, 216, 217*)
4.	ATP	Deoxy-ADP[e]	— (yeast, liver, and kidney)	(*166, 219, 221*)
4a.	ATP	Deoxy-GDP[e]	—	(*166*)
4b.	ATP	Deoxy-CDP[e]	—	(*166, 218, 221*)
5.	ATP	TDP[e]	—	(*166, 167, 218, 221, 222*)

6.	ATP	Phosphorylated RNA[a]	Myokinase (rabbit muscle and guinea pig liver)	(*183*, *206*, *225*; but cf. *38a*)
7.	ITP	UDP	— (pig kidney)	(*212*)
8.	ITP	Deoxy-ADP	— (*Azotobacter* spp.)	(*221*)

[a] Probably:



$$\mathrm{ATP} + \mathrm{R{-}O{-}}\underset{\mathrm{O{-}R'}}{\overset{\mathrm{O}}{\mathrm{P}}}\mathrm{{-}O{-}}\underset{\mathrm{OH}}{\overset{\mathrm{O}}{\mathrm{P}}}\mathrm{{-}OH} \rightleftarrows \mathrm{ADP} + \mathrm{R{-}O{-}}\underset{\mathrm{O{-}R'}}{\overset{\mathrm{O}}{\mathrm{P}}}\mathrm{{-}O{-}}\underset{\mathrm{OH}}{\overset{\mathrm{O}}{\mathrm{P}}}\mathrm{{-}O{-}}\underset{\mathrm{OH}}{\overset{\mathrm{O}}{\mathrm{P}}}\mathrm{{-}OH}$$

transfer reactions should not be overlooked. Such reactions, catalyzed by phosphatases, are shown in Table IV (*228–257a*).

Synthesis of organic phosphates by reversal of hydrolysis, catalyzed by the phosphomonoesterases, has often been demonstrated *in vitro* (Table IV, Group I). However, from the equilibrium data in Table I it is clear that no appreciable concentrations of phosphorylated metabolites could be formed in this way under physiological conditions.

The phosphomonoesterases of wide substrate specificity also catalyze phosphoryl transfer from organic phosphate donors to suitable acceptors. A number of such reactions are shown in Table IV, Group II, and it is seen that phosphoryl transfer occurs between a wide range of compounds. The acceptors must compete with water for the available phosphoryl groups, and some hydrolysis always accompanies formation of new phosphate esters. The relative amount of phosphoryl transfer to water (hydrolysis) and to the organic acceptor (transferase activity) is determined by the nature of the acceptor and of the enzyme. Since the extent of ester synthesis also depends on the relative concentrations of the organic acceptor and water, under most physiological conditions hydrolysis is predominant. However surprisingly high percentage transfers to pyrimidine nucleosides have been observed with enzymes from plant tissues (*244, 245, 258, 259*). All the hydroxyl groups in a metabolite are not phosphorylated to the same extent. Only glycerol-1-phosphate is formed during phosphoryl transfer to glycerol in the presence of alkaline phosphatase, and glucose is mainly phosphorylated at C-6 (*229, 238, 240*). It is apparent from Table IV that there is a remarkable specificity for the 5′-position of nucleosides. Formation of riboflavin phosphate from riboflavin by the transferase activity of alkaline phosphatase has recently been suggested as a physiologically important reaction in intestinal mucosa (*247*). This transferase activity of phosphatases illustrates the independence of reaction rates and free energy changes. In the presence of alkaline phosphatase, enolpyruvate phosphate, creatine phosphate, hexose diphosphate and glycerol-2-phosphate all phosphorylate glycerol to the same extent despite the great differences in their phosphoryl transfer potentials (*119, 229*). Table IV, Group II, includes a number of compounds of possible biological significance for which no kinases have yet been described.

Similar reactions involving transfer of esterified phosphoryl groups are catalyzed by phosphodiesterases, as shown in Table IV, Group III. The best known of these enzymes, pancreatic ribonuclease, is specific for the phosphate esters of pyrimidine nucleosides, while the corresponding diesterase from spleen catalyzes transfer from both purine and pyrimidine nucleoside phosphates (*252, 253*). Pancreatic ribonuclease can catalyze polymer formation from pyrimidine nucleoside phosphates, but the physiological significance of these observations is not known (*253*). Lecithin formation (Table IV, Group IV) transfer of phosphoryl choline from cytidine diphosphocholine to diglycerides is formally similar to the phosphoester transfers discussed above, but takes place without hydrolysis of the phosphoanhydride (*255, 256*).

3. *Phosphoryl Transfer Catalyzed by Mutases*

A number of essential metabolic sequences involve the interconversion of isomeric phosphate esters (e.g., of glucose-1-phosphate and glucose-6-phosphate in glycolysis). Originally these were thought to be intramolecular rearrangements, but it has now been established that they involve phosphoryl transfer between different molecules. In each case the corresponding diphosphate is an essential intermediate. Thus in the case of phosphoglucomutase the reaction is:

$$\text{Glucose-1-phosphate} + \text{Glucose-1,6-diphosphate} \rightleftarrows \text{Glucose-1,6-diphosphate} + \text{Glucose-6-phosphate}$$

This reaction is known to involve a phosphorylated enzyme, as discussed above (Section II,C,8) (*19, 280*). However the interconversion of glycerate-2-phosphate and glycerate-3-phosphate does not appear to involve a similar phosphorylated enzyme (*264, 270*). As in the case of kinases, closely related compounds are frequently substrates for the one enzyme (*250, 273, 279*). The mutase reactions are shown in Table V, see p. 60 (*260–283*).

4. *Transfer of Pyrophosphoryl Groups from ATP*

Since the pyrophosphate transfer potential of ATP is even greater than the phosphate transfer potential (cf. Table II) it might be expected that this nucleotide would serve as a donor of pyrophosphoryl groups in metabolism. Two reactions of this kind are known (Table VI[*]). The formation of thiamine pyrophosphate (*289*) (cocarboxylase) and of the important ribotide donor 5-phosphoribosyl-1-pyrophosphate (*286*), involve pyrophosphoryl transfer from ATP. The latter reaction prepares ribose-5-phosphate for subsequent participation in pyrophosphorylase reactions. The metabolic significance of the pyrophosphate group in thiamine pyrophosphate is at present unknown. It does not appear to function as a phosphoryl donor. The corresponding mono and triphosphates have little coenzyme activity (*290*).

B. PHOSPHOROLYSIS AND PYROPHOSPHOROLYSIS

These reactions involve transfer of phosphate and pyrophosphate groups. The O—P bond of phosphate (*111, 291*) or pyrophosphate

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[*] References *284–289* are in Table VI, see p. 62.

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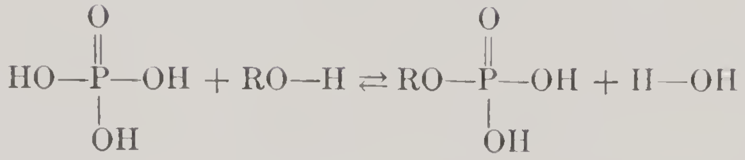
TABLE IV
PHOSPHORYL TRANSFER FROM PHOSPHOMONOESTERS AND RELATED COMPOUNDS CATALYZED BY PHOSPHATASE

GROUP I. TRANSFER FROM INORGANIC PHOSPHATE (REVERSAL OF HYDROLYSIS)[a]



$$HO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-OH + RO-H \rightleftharpoons RO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-OH + H-OH$$

		References	
	Acceptors	Acid phosphomonoesterases	Alkaline phosphomonoesterases
1.	Methanol		*(228)*
2.	Glycerol (at C-1 and C-2)	*(13)*	
3.	Glycerol (at C-1)	*(8)*	*(8, 10–12)*
3a.	Glycerate (at C-3)		*(14)*
4.	Glucose (at C-6)	*(8)*	*(8, 10, 229)*
5.	Mannose (at C-6)		*(8)*
6.	Fructose (at C-1)	*(8)*	*(8)*
7.	Fructose (at C-6)	*(8)*	*(8)*
8.	Galactose (at C-6)		*(8)*
9.	Cytidine (at C-2′, C-3′, C-5′)	*(230)*	
10.	Choline		*(231)*
10a.	Ethanolamine		*(231)*

Group II. Phosphotransferase reactions[b]

		Donor $\mathrm{RO}-\overset{\overset{\displaystyle \mathrm{O}}{\|}}{\underset{\underset{\displaystyle \mathrm{OH}}{\|}}{\mathrm{P}}}-\mathrm{OH}$	Acceptor $\overset{\overset{\displaystyle \mathrm{R'}}{\|}}{\underset{\underset{\displaystyle \mathrm{R''}}{\|}}{\mathrm{CHOH}}}$	References	
				Acid phosphomonoesterases	Alkaline phosphomonoesterases
1.	a.	Phenolphthalein phosphate	Methanol	*(232)*	
	b.	Phenol phosphate	Methanol	*(233, 234)*	
	c.	*p*-Nitrophenol phosphate	Methanol	*(235)*	
2.	a.	Phenolphthalein phosphate	Ethanol	*(232)*	
	b.	Phenol phosphate	Ethanol	*(233)*	
3.	a.	Phenolphthalein phosphate	*n*-Propanol	*(232)*	
	b.	*p*-Nitrophenol phosphate	*n*-Propanol	*(236)*	
	c.	Phenol phosphate	*n*-Propanol	*(233)*	
4.	a.	*p*-Nitrophenol phosphate	Isopropanol	*(236)*	
	b.	Phenolphthalein phosphate	Isopropanol	*(232)*	
5.		*p*-Nitrophenol phosphate	*n*-Butanol	*(236)*	
6.		*p*-Nitrophenol phosphate	Isobutanol	*(236)*	
7.		*p*-Nitrophenol phosphate	*sec*-Butanol	*(236)*	
8.		*p*-Nitrophenol phosphate	Allyl alcohol	*(236)*	
9.		*p*-Nitrophenol phosphate	Nitroethanol	*(236)*	
10.		Phenolphthalein phosphate	Ethylene glycol	*(232)*	
11.		*p*-Nitrophenol phosphate	Ethylene glycol	*(237)*	
12.	a.	*p*-Nitrophenol phosphate	Propylene glycol	*(236, 237)*	

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[a] Reactions 1 to 40 appear to be catalyzed by unspecific acid and alkaline phosphomonoesterases. The acid phosphatases were obtained from chicken, rat, and pigeon liver; human urine, red cells, prostate; snake venom; takadiastase; and a variety of plant tissues, particularly citrus, apple, carrot, and wheat.

[b] Phosphoryl transfer to organic acceptors is accompanied by hydrolysis; where known the principal position of phosphorylation is indicated. Small amounts of other isomers are often formed.

TABLE IV, GROUP II (*Continued*)

		Donor $\mathrm{RO{-}\underset{\underset{OH}{\vert}}{\overset{\overset{O}{\Vert}}{P}}{-}OH}$	Acceptor $\mathrm{\underset{\underset{R''}{\vert}}{\overset{\overset{R'}{\vert}}{CHOH}}}$	References	
				Acid phosphomonoesterases	Alkaline phosphomonoesterases
	b.	Creatine phosphate	Propylene glycol	*(238)*	*(229)*
13.		Creatine phosphate	Glyceraldehyde (at C-3)	*(238)*	*(229)*
14.		Creatine phosphate	Dihydroxyacetone (at C-1)	*(238)*	*(229)*
15.	a.	*p*-Nitrophenol phosphate	Glycerol (at C-1)	*(233, 235–237, 239)*	*(229, 239)*
	b.	Creatine phosphate	Glycerol (at C-1)	*(238, 239)*	*(119, 229, 239, 240)*
	c.	Phenol phosphate	Glycerol (at C-1)	*(241)*	*(241)*
	d.	Enolpyruvate phosphate	Glycerol (at C-1)	*(239)*	*(119, 229, 239, 240)*
	e.	Acetyl phosphate	Glycerol (at C-1)	*(239)*	
	f.	Glucose-1-phosphate	Glycerol (at C-1)		*(240)*
	g.	Fructose-1,6-diphosphate	Glycerol (at C-1)		*(120, 229)*
	h.	Glycerol-2-phosphate	Glycerol (at C-1)		*(119, 229)*
16.		*p*-Nitrophenol phosphate	Erythritol	*(236)*	
17.		*p*-Nitrophenol phosphate	Mannitol	*(236)*	
18.		Acetyl phosphate	Ribose	*(239)*	
19.	a.	Enolpyruvate phosphate	Glucose	*(240)*	
	b.	Creatine phosphate	Glucose (at C-6)	*(119, 238)*	*(119, 120, 229)*
	c.	Acetyl phosphate	Glucose	*(239)*	
	d.	*p*-Nitrophenol phosphate	Glucose	*(235, 237)*	
	e.	Phenol phosphate	Glucose (at C-6)	*(234, 241)*	*(119, 229, 241)*
	f.	Phenolphthalein phosphate	Glucose (at C-6)		*(229)*
	g.	Adenosine-5′-phosphate	Glucose (at C-6)		*(229)*
	h.	Glycerol-2-phosphate	Glucose (at C-6)	*(238)*	*(119, 229)*

20.	a.	Acetyl phosphate	Fructose (at C-1 and C-6)	*(239)*	
	b.	Creatine phosphate	Fructose (at C-1 and C-6)		*(240)*
			Fructose (at C-6)		*(229)*
	c.	Phenol phosphate	Fructose	*(234)*	
21.		Acetyl phosphate	Trehalose	*(239)*	
22.		Serine phosphate	Serine[c]	*(242, 243)*[b]	
23.		*p*-Nitrophenol phosphate	Ethyl lactate	*(236)*	
24.		*p*-Nitrophenol phosphate	Ethylene chlorohydrin	*(236)*	
25.		Phenol phosphate	Adenosine (at C-5′)	*(244)*	
26.		Phenol phosphate	Deoxyadenosine (at C-5′)	*(244)*	
27.		Phenol phosphate	Guanosine[d]	*(244)*	
28.		Phenol phosphate	Deoxyguanosine[e]	*(244)*	
29.		Phenol phosphate	Inosine (at C-5′)	*(244)*	
30.		Phenol phosphate	Deoxyinosine (at C-5′)	*(244)*	
31.	a.	Phenol phosphate	Uridine (at C-5′)	*(244, 245)*	
	b.	Cytidine-5′-phosphate	Uridine (at C-5′)	*(244)*	
	c.	Cytidine-3′(and 2′)-phosphate	Uridine (at C-5′)	*(244)*	
	d.	Deoxycytidine phosphate	Uridine (at C-5′)	*(244)*	
32.		Phenol phosphate	Deoxyuridine (at C-5′)	*(244)*	
33.	a.	Phenol phosphate	Cytidine[d]	*(244)*	
	b.	Adenosine-5′-phosphate	Cytidine[d]	*(244)*	
	c.	Adenosine-3′-phosphate	Cytidine (at C-5′)	*(244)*	
	d.	ATP	Cytidine (at C-5′)[f]	*(244)*	
	e.	Ribose-5-phosphate	Cytidine[d]	*(244)*	
	f.	Glucose-1-phosphate	Cytidine	*(244)*	
	g.	Glycerol-2-phosphate	Cytidine	*(244)*	

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[c] This reaction is catalyzed by serine phosphatase.

[d] Phosphorylation at C-5′ with plant phosphotransferase enzyme, at C-2′ and C-3′ with liver enzyme, and at C-2′, C-3′, and C-5′ with prostate enzyme.

[e] Phosphorylation at C-5′ with plant enzyme, at C-3′ with liver enzyme, and at C-3′ and C-5′ with prostate enzyme.

[f] Possibly formed by phosphoryl transfer from AMP.

TABLE IV, GROUP II (*Continued*)

		Donor $RO{-}\overset{\overset{O}{\|}}{\underset{\underset{OH}{\mid}}{P}}{-}OH$	Acceptor $\overset{\overset{R'}{\mid}}{\underset{\underset{R''}{\mid}}{CHOH}}$	References	
				Acid phosphomonoesterases	Alkaline phosphomonoesterases
	h.	Inosine-5′-phosphate	Cytidine (at C-5′)	(*244*)	
	i.	Deoxyadenosine phosphate	Cytidine (at C-5′)	(*244*)	
34.		Phenol phosphate	Deoxycytidine	(*244*)	
35.		Phenol phosphate	Thymidine	(*244*)	
36.		Phenol phosphate	Orotidine (at C-5′)	(*77*)	
37.		Phenol phosphate	8-Azaguanosine	(*246*)	
38.		Phenol phosphate	Nucleoside-2′-phosphate (at C-5′)	(*245*)	
39.		Phenol phosphate	Adenosine (2′,3′)-diphosphate (at C-5′)	(*245*)	
40.	a.	Glycerol-1-phosphate	Riboflavin (at C-5′)		(*247*)
	b.	Phenol phosphate	Riboflavin (at C-5′)		(*247*)
	c.	Adenosine-5′-phosphate	Riboflavin (at C-5′)		(*247*)
41.		Glucose-1-phosphate	Glucose-1-phosphate (at C-6)^g		(*248–251*)

GROUP III. PHOSPHOTRANSFERASE REACTIONS CATALYZED BY PHOSPHODIESTERASES

		Donor $RO{-}\overset{\overset{O}{\|}}{\underset{\underset{\underset{\underset{R'}{\mid}}{O}}{\mid}}{P}}{-}OH$	Acceptor $R''{-}\underset{\underset{R'''}{\mid}}{CHOH}$	Diester product	References
1.	a.	Benzyl-P-3′-cytidine	Methanol	Methyl-P-3′-cytidine^{h,i}	(*252*)
	b.	Cytidine-2′,3′-P	Methanol	Methyl-P-3′-cytidine^h	(*252*)
	c.	Uridine-2′,3′-P	Methanol	Methyl-P-3′-uridine^h	(*252*)

	d.	Cytidine-2′,3′-P	Ethanol	Ethyl-P-3′-cytidine^h	(*252*)
	e.	Uridine-2′,3′-P	Ethanol	Ethyl-P-3′-uridine^h	(*252*)
	f.	RNA	Methanol	Methyl-P-3′-cytidine^h and Methyl-P-3′-uridine	(*252*)
2.	a.	Cytidine-2′,3′-P	Adenosine	5′-Adenyl-P-3′-cytidine^h	(*253*)
	b.	Cytidine-2′,3′-P	Cytidine	5′-Cytidyl-P-3′-cytidine^h	(*253*)
	c.	Uridine-2′,3′-P	Cytidine	5′-Cytidyl-P-3′-uridine^h	(*253*)
3.	a.	Benzyl-P-3′-adenosine	Methanol	Methyl-P-3′-adenosine^i	(*252*)
	b.	Benzyl-P-3′-adenosine	Ethanol	Ethyl-P-3′-adenosine^i	(*252*)
	c.	5′-Uridyl-P-3′-adenosine	Methanol	Methyl-P-3′-adenosine^i	(*252*)
	d.	5′-Uridyl-P-3′-adenosine	Ethanol	Ethyl-P-3′-adenosine^i	(*252*)
	e.	RNA "core"	Methanol	Methyl-P-3′-adenosine^i and Methyl-P-3′-guanosine	(*252*)
4.	a.	Ethyl-P-*p*-nitrophenol	Glycol	Ethyl-P-glycol^j	(*254*)
	b.	Ethyl-P-*p*-nitrophenol	Glycerol	Ethyl-P-glycerol^j	(*254*)
5.		Cytidine-2′,3′-P	Adenosine-2′,3′-P	2′,3′-P-5′-adenyl-3′-cytidine^h	(*253*)
6.		5′-Uridyl-P-3′-adenosine	Cytidine	5′-Cytidyl-P-3′-adenosine^i	(*253*)
7.	a.	Benzyl-P-3′-cytidine	Benzyl-P-3′-cytidine	3′-Benzyl-P-5′-cytidyl-P-3′-cytidine^i	(*253*)
	b.	Benzyl-P-3′-adenosine	Benzyl-P-3′-adenosine	3′-Benzyl-P-5′-adenyl-P-3′-adenosine^i	(*253*)
8.		Cytidine-3′-P (internal transfer)	—	Cytidine-2′,3′-P^h	(*253*)

---

[g] Probably catalyzed by a specific enzyme from *E. coli* and skeletal muscle.

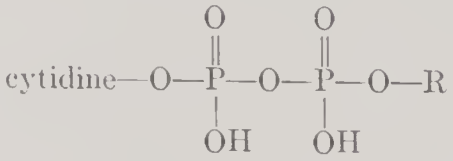
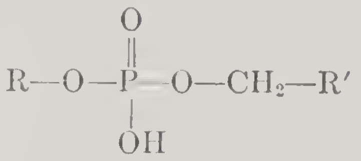
[h] Catalyzed by pancreatic ribonuclease.

[i] Catalyzed by spleen diesterase.

[j] Catalyzed by phosphodiesterase of snake venom, rat, rabbit, and pig kidney, and ox and rat liver.

TABLE IV (*Continued*)

GROUP IV. SPECIFIC PHOSPHOESTER TRANSFER FROM PHOSPHOANHYDRIDES[k]

	Donor  $\text{cytidine—O—}\overset{\displaystyle O}{\underset{\displaystyle OH}{\overset{\Vert}{\underset{\vert}{P}}}}\text{—O—}\overset{\displaystyle O}{\underset{\displaystyle OH}{\overset{\Vert}{\underset{\vert}{P}}}}\text{—O—R}$	Acceptor $HO—CH_2—R'$	Product  $R—O—\overset{\displaystyle O}{\underset{\displaystyle OH}{\overset{\Vert}{\underset{\vert}{P}}}}—O—CH_2—R'$	References
1.	CDP-choline	1,2-Diglycerides	Lecithins[l]	*(255, 256)*
2.	CDP-ethanolamine	1,2-Diglycerides	Cephalins[l]	*(256)*
3.	CDP-ribitol	Teichoic acid	Teichoic acid polymer[m]	*(257)*
4.	CDP-phosphatide	*myo*-Inositol[n]	Inositol phosphatide	*(257a)*

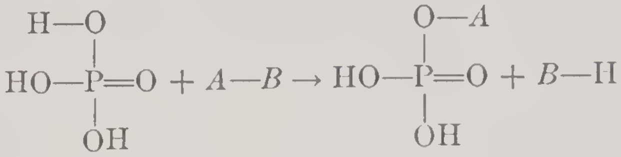
[k] This phosphomonoester transfer is not accompanied by hydrolysis.

[l] Demonstrated in brain, liver, and nerve.

[m] Demonstrated in *Staphylococcus aureus*, *Bacillus subtilis*, and *Lactobacillus arabinosus*.

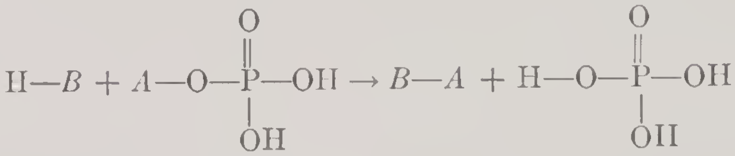
[n] Secondary —OH in acceptor.

(*291, 292*) remains intact. This is shown below for the reactions catalyzed by phosphorylases:



$$\mathrm{HO{-}}\overset{\mathrm{H{-}O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{=}O} + A{-}B \rightarrow \mathrm{HO{-}}\overset{\mathrm{O{-}}A}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{=}O} + B{-}\mathrm{H}$$

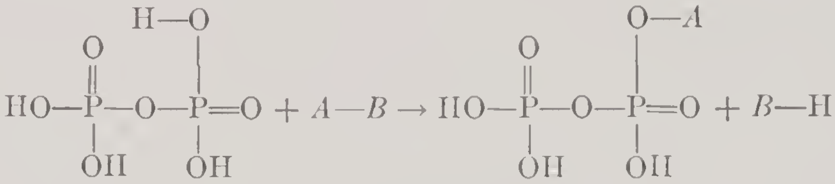
Phosphorolysis (nucleophilic displacement of *B* from *A* by phosphate O)



$$\mathrm{H{-}}B + A\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}OH} \rightarrow B{-}A + \mathrm{H{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}OH}$$

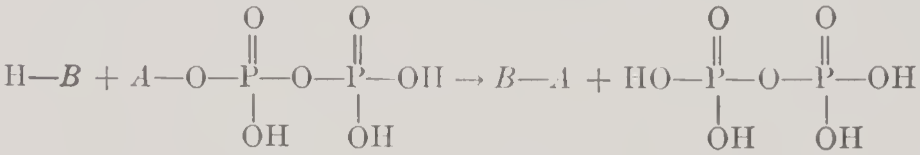
Reversal of phosphorolysis (nucleophilic displacement of phosphate from *A* by *B*)

and by pyrophosphorylases:



$$\mathrm{HO{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{H{-}O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{=}O} + A{-}B \rightarrow \mathrm{HO{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{O{-}}A}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{=}O} + B{-}\mathrm{H}$$

Pyrophosphorolysis

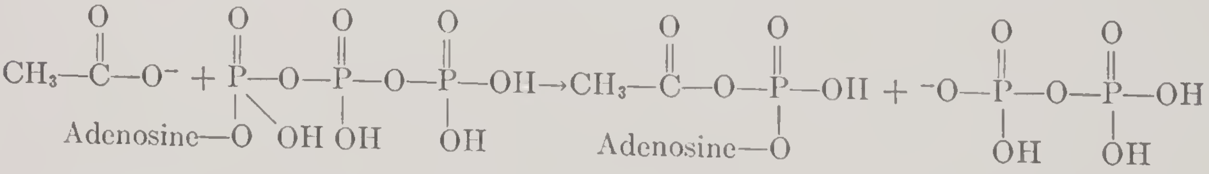


$$\mathrm{H{-}}B + A\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}OH} \rightarrow B{-}A + \mathrm{HO{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}OH}$$

Reversal of pyrophosphorolysis

Both forward and reverse reactions involve nucleophilic displacements from the same group (*A*). This is in contrast to the phosphoryl transfers catalyzed by kinases, phosphatases, and mutases, where the P—O bond is broken during nucleophilic attack on the phosphorus.

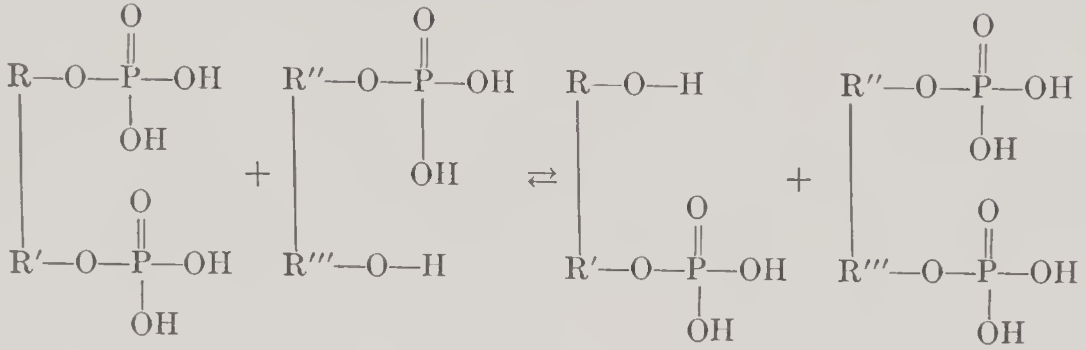
Phosphorylases and pyrophosphorylases thus catalyze transfer of the electrophilic group (*A*) between *B* and phosphate or pyrophosphate respectively. For instance in the activation of acetate by ATP



$$\mathrm{CH_3{-}}\overset{\mathrm{O}}{\mathrm{C}}\mathrm{{-}O^-} + \overset{\mathrm{O}}{\underset{\mathrm{Adenosine{-}O}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}OH} \rightarrow \mathrm{CH_3{-}}\overset{\mathrm{O}}{\mathrm{C}}\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{Adenosine{-}O}}{\mathrm{P}}}\mathrm{{-}OH} + \mathrm{^-O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}O{-}}\overset{\mathrm{O}}{\underset{\mathrm{OH}}{\mathrm{P}}}\mathrm{{-}OH}$$

the nucleophilic acetate displaces nucleophilic pyrophosphate from the electrophilic α-phosphorus of ATP. Thus an adenyl group is transferred from pyrophosphate to acetate, forming adenyl acetate. For this reason, pyrophosphorylases which catalyze this type of displacement have been called nucleotidyl transferases (*88*). Since in both directions these reactions involve transfer of phosphate or pyrophosphate groups, the more general terms phosphorylase and pyrophosphorylase are con-

TABLE V
Phosphoryl Transfer Catalyzed by Mutases



	Reactants	Products	References
1.	Glycerate-2-phosphate + Glycerate-2,3-diphosphate	Glycerate-2,3-diphosphate[a] + Glycerate-3-phosphate	*(17, 260–264)*
2.	Glycerate-1,3-diphosphate + Glycerate-3-phosphate	Glycerate-3-phosphate[b] + Glycerate-2,3-diphosphate	*(262, 265–270)*
3.	Ribose-1,5-diphosphate + Ribose-5-phosphate	Ribose-1-phosphate[c] + Ribose-1,5-diphosphate	*(271–275)*
3a.	Deoxyribose-1,5-diphosphate + Deoxyribose-5-phosphate	Deoxyribose-1-phosphate[d] + Deoxyribose-1,5-diphosphate	*(276)*
4.	Glucose-1,6-diphosphate + Glucose-6-phosphate	Glucose-1-phosphate[e] + Glucose-1,6-diphosphate	*(19, 21, 277–282)*

5.	*N*-Acetylglucosamine-1,6-diphosphate + *N*-Acetylglucosamine-6-phosphate	*N*-Acetylglucosamine-1-phosphate[f] + *N*-Acetylglucosamine-1,6-diphosphate	*(146)*
6.	Glucosamine-1,6-diphosphate + Glucosamine-6-phosphate	Glucosamine-1-phosphate[g] + Glucosamine-1,6-diphosphate	*(283)*
7.	Mannose-1,6-diphosphate + Mannose-6-phosphate	Mannose-1-phosphate[h] + Mannose-1,6-diphosphate	*(279)*

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[a] Demonstrated in yeast, rabbit muscle, and erythrocytes.

[b] Demonstrated in rabbit, pig and chicken blood and in rabbit, rat, sheep and beef tissues.

[c] Demonstrated in bakers' and brewers' yeast and in rabbit and bovine muscle. Also present in spleen, brain, and blood.

[d] Demonstrated in rat liver and calf thymus.

[e] Demonstrated in yeast, rabbit muscle, and rat liver.

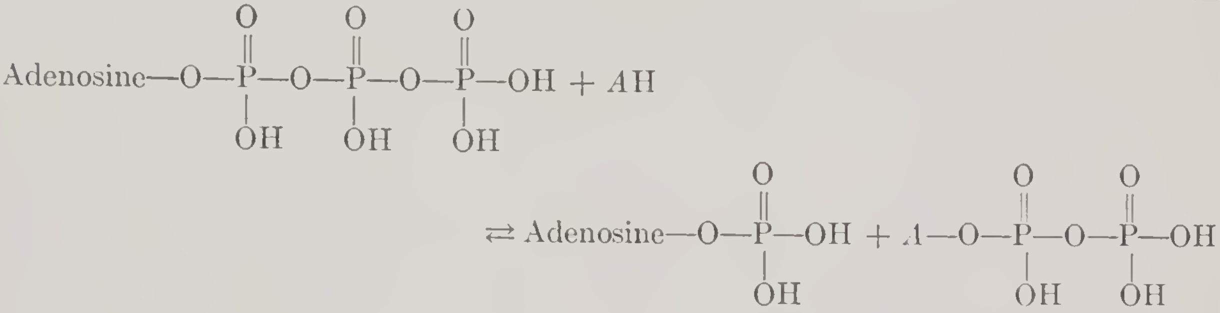
[f] Demonstrated in *Neurospora crassa.*

[g] Demonstrated in rabbit muscle.

[h] Probably identical with phosphoglucomutase.

venient, and have been used here. It seems likely that in many of these reactions pyrophosphate is first displaced by a nucleophilic group of the enzyme, forming a nucleotidyl-enzyme intermediate from which the enzyme is then displaced by the nucleophilic reactant *B*. However there is still uncertainty about details of the mechanism of these reactions (*89*).

TABLE VI
Pyrophosphoryl Transfer from ATP



$$\text{Adenosine—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—OH} + A\text{H} \rightleftarrows \text{Adenosine—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—OH} + A\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—O—}\underset{\text{OH}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}\text{—OH}$$

Acceptor	References
1. α-D-Ribose-5-phosphate (at C-5)[a]	(*89, 284–286*)
2. Thiamine[b]	(*89, 287–289*)

[a] Demonstrated in *E. coli*, pigeon, chicken, mouse, rat, and beef liver.
[b] Found in rat liver and yeast.

1. *Reactions Catalyzed by Phosphorylases*

Physiologically it is essential that these reactions occur in both directions. As seen from Table VII,[*] there are four types of reactions catalyzed by phosphorylases. The first group involves phosphorolysis of the glycosidic C—O—C linkage of carbohydrates. The reaction catalyzed by muscle phosphorylase is typical:

$$\text{Glycogen} + \text{P}_i \rightleftarrows \text{Glucose-1-phosphate.}$$

This permits interconversion of the reactive intermediate glucose-1-phosphate and the storage polysaccharide, glycogen. As seen from Table I, the equilibrium constant is close to unity, and the direction in which the reaction proceeds is dependent on the relative concentrations of glucose-1-phosphate and of inorganic phosphate. In almost all known cases the configuration of the glycosidic carbon is the same in the phosphate (e.g., α-D-glucose-1-phosphate) and in the polyglycoside (e.g., α-D-glucosyl polyglycoside; glycogen or starch). Several authors have recently discussed possible mechanisms involved in these displacements (*89, 299, 364*).

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[*] References *293–363* are in Table VII.

The second type of reaction is the reversible phosphorolysis of *N*-glycosides, e.g.,

$$\underset{\text{(Uridine)}}{\text{Uracil-}\beta\text{-}N\text{-riboside}} + P_i \rightleftarrows \text{Uracil} + \alpha\text{-D-Ribose-1-phosphate}$$

Since most nucleosides can be phosphorylated to nucleotides by ATP in the presence of appropriate kinases (cf. Table III), these reactions can be steps in the formation of nucleotides:

$$\text{Base} + \text{Ribose-1-phosphate} \xrightleftharpoons{\text{(phosphorylase)}} \text{Nucleoside} + P_i$$

$$\text{Nucleoside} + \text{ATP} \xrightleftharpoons{\text{(kinase)}} \text{Nucleotide} + \text{ADP}$$

However, as recently pointed out by Kornberg (*166*), phosphorylases probably have an important function in the "salvage" of heterocyclic bases of nucleotides which have been dephosphorylated in metabolism:

$$\text{Nucleotide} + \text{Water} \xrightleftharpoons{\text{(phosphatase)}} \text{Nucleoside} + P_i$$

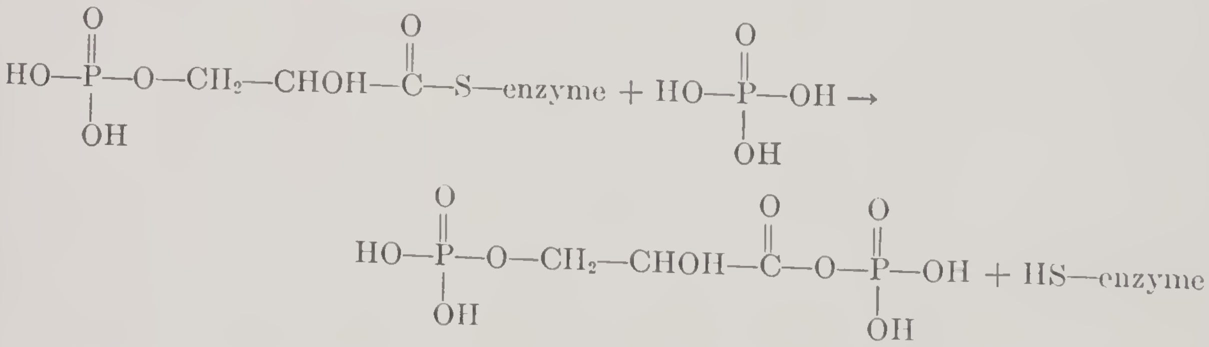
$$\text{Nucleoside} + P_i \xrightleftharpoons{\text{(phosphorylase)}} \text{Ribose-1-phosphate} + \text{Base}$$

$$\text{Base} + \text{5-Phosphoribosyl-1-pyrophosphate} \xrightleftharpoons{\text{(pyrophosphorylase)}} \text{Nucleotide} + PP_i$$

As shown above, phosphorolysis of nucleosides involves the formation of α-1-glycosyl phosphates from β-N-glycosides.

Carbamyl transfer (Table VII, Group II) is also a reaction involving phosphorolysis of a C—N bond. Here also reversibility is physiologically important.

Phosphorolysis of thiol esters (Table VII, Group III) results in the formation of phosphoanhydrides, e.g.



$$HO-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O-CH_2-CHOH-\overset{\overset{O}{\|}}{C}-S-\text{enzyme} + HO-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-OH \rightarrow$$

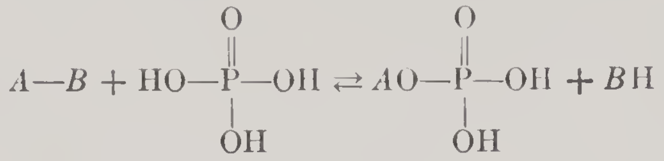
$$HO-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O-CH_2-CHOH-\overset{\overset{O}{\|}}{C}-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-OH + HS-\text{enzyme}$$

In this way inorganic phosphate is converted into an organic phosphate of high phosphoryl transfer potential. This reaction, which occurs during glycolysis, is reversible.

A particularly interesting phosphorylase which has recently been described catalyses reversible polynucleotide formation from nucleoside diphosphates:

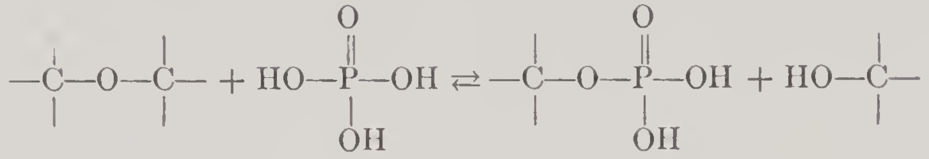
$$\text{Polyribonucleotide} + P_i \rightleftarrows \text{Ribonucleoside-5-diphosphates}$$

TABLE VII
Phosphorylase Reactions



$$A\text{—}B + \text{HO—}\underset{\text{OH}}{\underset{|}{\overset{\text{O}}{\overset{\|}{\text{P}}}}}\text{—OH} \rightleftarrows A\text{O—}\underset{\text{OH}}{\underset{|}{\overset{\text{O}}{\overset{\|}{\text{P}}}}}\text{—OH} + B\text{H}$$

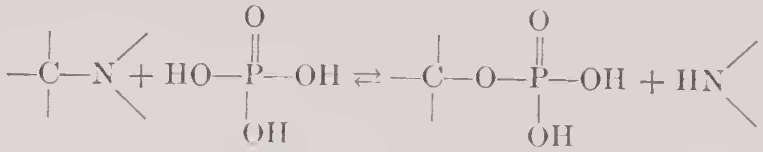
Group I.



$$\text{—}\underset{|}{\overset{|}{\text{C}}}\text{—O—}\underset{|}{\overset{|}{\text{C}}}\text{—} + \text{HO—}\underset{\text{OH}}{\underset{|}{\overset{\text{O}}{\overset{\|}{\text{P}}}}}\text{—OH} \rightleftarrows \text{—}\underset{|}{\overset{|}{\text{C}}}\text{—O—}\underset{\text{OH}}{\underset{|}{\overset{\text{O}}{\overset{\|}{\text{P}}}}}\text{—OH} + \text{HO—}\underset{|}{\overset{|}{\text{C}}}\text{—}$$

A—B	Source	References
1. α-D-Glucosylarabinopyranoside	Catalyzed by sucrose phosphorylase present in *Pseudomonas saccharophila* and *Leuconostoc mesenteroides.*	(*293*, *294*; cf. ref. *295*)
2. α-D-Glucosylarabofuranoside	Catalyzed by sucrose phorphorylase present in *Pseudomonas saccharophila* and *Leuconostoc mesenteroides.*	(*295*)
3. α-D-Glucosylxylose	Catalyzed by sucrose phosphorylase present in *Pseudomonas saccharophila* and *Leuconostoc mesenteroides.*	(*67*, *294*)
4. Maltose (to β-D-glucose-1-phosphate + Glucose)	Occurs in *Neisseria meningitidis.*	(*67*, *294*)
5. α-D-Glucosylsorbofuranoside	Catalyzed by sucrose phosphorylase present in *Pseudomonas saccharophila* and *Leuconostoc mesenteroides.*	(*293*, *294*; cf. ref. *295*)
6. Sucrose (to α-D-glucose-1-phosphate + fructose)	Catalyzed by sucrose phosphorylase present in *Pseudomonas saccharophila* and *Leuconostoc mesenteroides.*	(*293*, *294*, *296*, *297*)
7. Glucosyl-(sucrose phosphorylase)	Catalyzed by sucrose phosphorylase present in *Pseudomonas saccharophila* and *Leuconostoc mesenteroides.*	(*293*, *296*, *298*; cf. *295*, *299*)
8. Glycogen and starch (to α-D-glucose-1-phosphate)	Widely distributed in plants and animals.	(*69–71*, *111*, *300–307*)

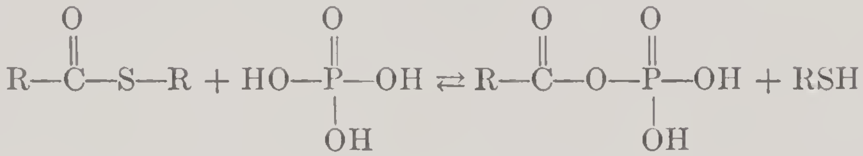
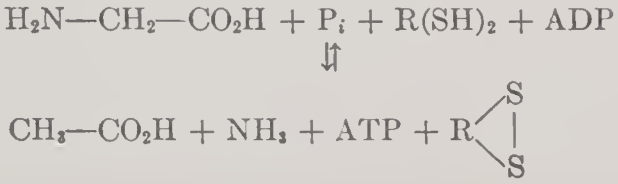
Group II.



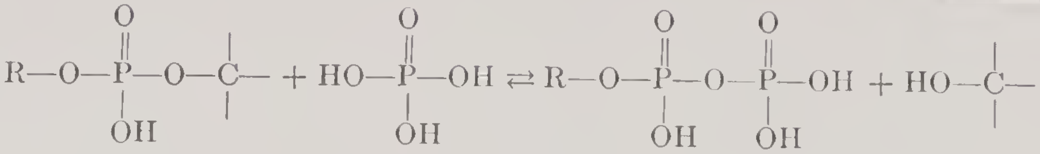
$$-\overset{|}{\underset{|}{C}}-N\!\langle + HO-\overset{O}{\overset{\|}{\underset{\underset{OH}{|}}{P}}}-OH \rightleftharpoons -\overset{|}{\underset{|}{C}}-O-\overset{O}{\overset{\|}{\underset{\underset{OH}{|}}{P}}}-OH + HN\!\langle$$

A—B	Source	References
1. Adenosine	Occurs in *E. coli* and liver.	(*308–312*)
2. Guanosine	Occurs in bakers' yeast.	(*88, 313, 314*)
3. Inosine	Occurs in bakers' yeast.	(*312–317*)
4. Xanthosine	Found in rat liver.	(*318*)
5. Azaguanine riboside	Occurs in horse liver.	(*319*)
6. 2,6-Diaminopurine riboside	Found in beef liver.	(*311*)
7. Uridine	Occurs in rat liver.	(*166, 320–322*)
8. Deoxyguanosine	Occurs in *E. coli* and liver.	(*75, 316, 323*)
9. Deoxyinosine	Occurs in *E. coli* and liver.	(*75, 276, 316, 323*)
10. Azaguanine deoxyriboside	Occurs in horse liver.	(*319*)
11. Deoxyxanthosine	Found in rat liver.	(*318*)
12. Deoxyuridine	Occurs in horse liver.	(*324, 325*)
13. Deoxycytidine	Occurs in *E. coli* and liver.	(*323*)
14. Thymidine	Occurs in horse liver.	(*323–325*)
15. 4-Aminoimidazole-5-carboxamide riboside	Found in beef liver.	(*309, 311*)
16. Nicotinamide riboside	Found in beef liver.	(*74*)
17. 5-Aminodeoxyuridine	Occurs in horse liver.	(*325*)
18. 5-Iododeoxyuridine	Occurs in horse liver.	(*325*)
19. 2-Thiodeoxyuridine	Occurs in horse liver.	(*325*)
20. 2-Bromodeoxyuridine	Occurs in horse liver.	(*325*)

TABLE VII (*Continued*)

A—B	Source	References
21. 5-Iodo-2-thiodeoxyuridine	Occurs in horse liver.	(*325*)
22. 5-Bromo-2-thiodeoxyuridine	Occurs in horse liver.	(*325*)
23. 5-Chlorodeoxyuridine	Occurs in horse liver.	(*325*)
24. Citrulline (to carbamyl phosphate + ornithine)	Occurs in rat liver, *Streptococcus faecalis*, *Streptococcus lactis*, and *E. coli*.	(*107*, *326–331*)
25. Carbamyl aspartate (to carbamyl phosphate and aspartate)	Occurs in rat liver, stomach, and intestine; also in *Streptococcus faecalis*.	(*107*, *332*)
GROUP III.	 $R—\overset{\overset{O}{\|}}{C}—S—R + HO—\underset{\underset{OH}{\vert}}{\overset{\overset{O}{\|}}{P}}—OH \rightleftarrows R—\overset{\overset{O}{\|}}{C}—O—\underset{\underset{OH}{\vert}}{\overset{\overset{O}{\|}}{P}}—OH + RSH$	
1. Acetyl coenzyme A	Occurs in *E. coli* and *Clostridium kluyveri*.	(*47*, *53*, *333*, *334*)
1a. Succinyl coenzyme A	Found in spinach, pig kidney cortex, heart, and liver.	(*125*, *335*, *336*)
2. 3-Phosphoglyceroyl-*S*-(phosphoglyceraldehyde dehydrogenase)	Occurs in yeast and rabbit muscle.	(*337–343*)
3. β-Aspartyl-*S*-(aspartaldehyde dehydrogenase)	Found in yeast.	(*344*)
4. 3-Phosphoglyceric methyl thioester (to 3-phosphoglyceroyl-P + CH_3SH)	Found in yeast.	(*129*)
5.  $H_2N—CH_2—CO_2H + P_i + R(SH)_2 + ADP$ ⇅ $CH_3—CO_2H + NH_3 + ATP + R$	Occurs in *Clostridium sticklandii*.	(*345*)

GROUP IV.



$$\mathrm{R{-}O{-}\underset{\displaystyle OH}{\underset{|}{\overset{\displaystyle O}{\overset{\|}{P}}}}{-}O{-}\underset{|}{\overset{|}{C}}{-} + HO{-}\underset{\displaystyle OH}{\underset{|}{\overset{\displaystyle O}{\overset{\|}{P}}}}{-}OH \rightleftarrows R{-}O{-}\underset{\displaystyle OH}{\underset{|}{\overset{\displaystyle O}{\overset{\|}{P}}}}{-}O{-}\underset{\displaystyle OH}{\underset{|}{\overset{\displaystyle O}{\overset{\|}{P}}}}{-}OH + HO{-}\underset{|}{\overset{|}{C}}{-}}$$

A—B	Source	References
1. Polyribonucleotide (to nucleoside diphosphate)	Occurs in yeast, *Azobacter vinelandii*, *Micrococcus lysodeikticus*, *E. coli*, spinach.	(*166*, *221*, *346–357*)
GROUP V.	PHOSPHOROLYSES OF UNKNOWN MECHANISM	
1. Xylulose-5-phosphate (to acetyl phosphate + glyceraldehyde-3-phosphate)	Occurs in *Lactobacillus pentosus* and *Acetobacter xylinum*.	(*358*, *359*)
1a. Fructose-6-phosphate (to acetyl phosphate + erythrose-4-phosphate)	Occurs in *Acetobacter xylinum*.	(*359*)
2. Pyruvate (to acetyl phosphate + formic acid)	Occurs in *Clostridium butylicum*, *Micrococcus lactilyticus*, and *E. coli*.	(*360*, *361*)
3. Pyruvate (to acetyl phosphate + hydrogen + carbon dioxide)	Occurs in *Clostridium butylicum*.	(*362*)
4. Pyruvate (to acetyl phosphate + hydrogen peroxide + carbon dioxide)	Occurs in *Lactobacillus delbrueckii*.	(*43*, *363*)

From a mixture of ADP, GDP, UDP, and CDP a polyribonucleotide is obtained which closely resembles natural ribonucleic acid (see Table VII, Group IV) (*355, 356*). Under physiological conditions, the amounts of mononucleotides and polynucleotides at equilibrium are similar (*349*), and this reaction may therefore account for the known rapid turnover of cytoplasmic RNA. There must be continual breakdown and resynthesis of this material in the living cell.

2. *Reactions Catalyzed by Pyrophosphorylases*

Of the major processes leading to the biosynthesis of phosphates this is the most recently discovered. The large number and diverse nature of reactions of this type already described is apparent from Table VIII. It seems likely that under most physiological conditions they take place in the direction of pyrophosphate displacement (i.e., reversal of pyrophosphorolysis). Many of the compounds formed by pyrophosphorylase action are essential coenzymes (e.g., diphosphopyridine nucleotide, coenzyme A, and flavin adenine dinucleotide) or reactive intermediates (e.g., uridine diphosphate glucose) which are common to many enzymatic pathways. Since pyrophosphate may be hydrolyzed to inorganic phosphate by inorganic pyrophosphatase (*432, 433, 434*), a mechanism is available to ensure that syntheses catalyzed by pyrophosphorylases proceed in the direction of pyrophosphate displacement. For instance, the successive reactions:

$$\text{Nicotinamide mononucleotide} + \text{ATP} \xrightleftharpoons{\text{(pyrophosphorylase)}} \text{DPN} + \text{PP}_i \qquad (\Delta F', +0.4 \text{ kcal./mole})$$

and

$$\text{PP}_i + \text{H}_2\text{O} \xrightleftharpoons{\text{(pyrophosphatase)}} 2\text{P}_i \qquad (\Delta F', -6.6 \text{ kcal./mole})$$

are together equivalent to the reaction

$$\text{NMN} + \text{ATP} + \text{H}_2\text{O} \rightleftarrows \text{DPN} + 2\text{P}_i \qquad (\Delta F', -6.2 \text{ kcal./mole})$$

Clearly, if both enzymes are present the reaction must proceed almost entirely in the direction of coenzyme synthesis.

The reactions in Table VIII, Group I, all result in formation of phosphoanhydrides, and involve displacement of pyrophosphate from an esterified phosphate.

The reactions in Group II of this table result in the formation of acyl adenylates, which are used in such metabolically important reactions as fatty acid oxidation, protein synthesis, and coenzyme formation.

All the reactions in Group III lead to nucleotide synthesis by pyrophosphate displacement from 5-phosphoribosyl-1-pyrophosphate. As indicated above this is probably the major pathway leading to nucleotides. Pyridine and pyrimidine nucleotide formation involves transfer of the 5-phosphoribosyl group to preformed cyclic compounds. In contrast, purine nucleotide biosynthesis takes place from 5-phosphoribosylamine, the purine ring being formed on the amino group of this compound. As in the case of nucleoside phosphorylases, the reactions catalyzed by nucleotide pyrophosphorylases take place with inversion at the glycosidic carbon, 5-phosphoribosyl-α-1-pyrophosphate being formed from the β-N-ribotides (*89, 286*).

Synthesis of polydeoxyribonucleotides occurs when a mixture of deoxyriboside-5′-triphosphates of adenine, guanine, cytosine, and thymine are incubated with pyrophosphorylases obtained from a number of microorganisms (Table VIII, Group IV). This may be the way in which deoxyribonucleic acid is formed in living cells. Removal of pyrophosphate by inorganic pyrophosphatase, perhaps after diffusion from the nucleus to the cytoplasm, would ensure that DNA was not broken down to mononucleotides by pyrophosphorolysis. The stability of DNA (*435, 436*) is in contrast to the rapid turnover of cytoplasmic RNA (*437*) and this difference may be due to their respective formation by pyrophosphorylase and phosphorylase action. These differences are consistent with present knowledge of the metabolic functions of the two kinds of nucleic acid (*435, 436, 437*).

There is at present little information about the reactions listed in Table VIII, Groups V and VI. The only one well established is the formation of adenylsulfate by displacement of the terminal pyrophosphate from ATP by sulfate. Further phosphorylation by a specific kinase (Table III) converts this compound into the active sulfate donor, 3-phosphoadenylsulfate (*177, 178, 430*).

The mechanisms of some reactions involving phosphorylated metabolites are still obscure, but there are few metabolites which cannot be formed by one or more of the reactions listed in Tables III to VIII.

The uridine diphosphate glycosides, and possibly other nucleoside diphosphate glycosides, are important glycosyl donors (*88, 257, 438, 108a*). Thus, sucrose synthesis in plants may occur as follows:

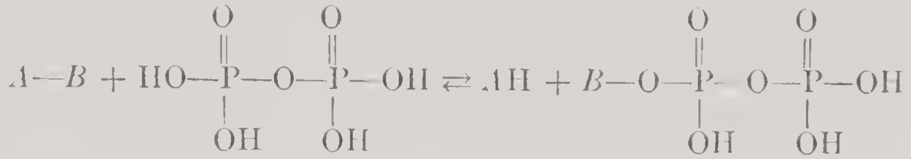
$$\text{UDPG} + \text{Fructose} \rightleftarrows \text{UDP} + \text{Sucrose} \quad (\textit{439})$$

IV. Phosphoryl Transfer Sequences in Metabolism

The synthesis of new materials such as protein, nucleic acid, lipid, and carbohydrate from smaller molecules is necessary for cell growth. As previously indicated, activation by ATP of compounds involved in

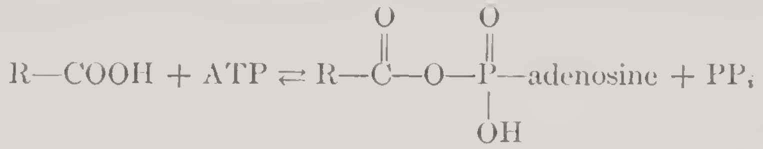
TABLE VIII

Pyrophosphorylase Reactions



$$A-B + HO-\overset{O}{\underset{OH}{P}}-O-\overset{O}{\underset{OH}{P}}-OH \rightleftarrows AH + B-O-\overset{O}{\underset{OH}{P}}-O-\overset{O}{\underset{OH}{P}}-OH$$

Group I. $R-O-\overset{O}{\underset{OH}{P}}-OH$	$R'-O-\overset{O}{\underset{OH}{P}}-O-\overset{O}{\underset{OH}{P}}-O-\overset{O}{\underset{OH}{P}}-OH$	$R-O-\overset{O}{\underset{OH}{P}}-O-\overset{O}{\underset{OH}{P}}-O-R'$	References
1. NMN	ATP	DPN^{a}	*(76, 365, 366)*
2. Nicotinic acid nucleotide	ATP	Desamido-DPN^{b}	*(367, 367a,b, 368)*
3. Phosphopantetheine	ATP	Dephosphocoenzyme A^{c}	*(175)*
4. Riboflavin phosphate	ATP	FAD^{d}	*(369)*
5. Xylose-1-phosphate	UTP	UDP-xylose^{e}	*(108a, 370, 371)*
5a. Arabinose-1-phosphate	UTP	UDP-arabinose^{e}	*(108a, 370)*
6. Glucose-1-phosphate	UTP	UDP-glucose^{f}	*(108a, 370, 372, 373)*
7. Galactose-1-phosphate	UTP	UDP-galactose^{g,gg}	*(108a, 370, 374)*
8. *N*-Acetylglucosamine-1-phosphate	UTP	UDP-*N*-acetylglucosamine^{h}	*(375)*
9. Mannose-1-phosphate	GTP	GDP-mannose^{g}	*(376–378)*
10. Glycerol-1-phosphate	CTP	CDP-glycerol^{i}	*(379)*
11. Ribitol-1-phosphate	CTP	CDP-ribitol^{i}	*(379)*
12. Choline phosphate	CTP	CDP-choline^{j,k}	*(255, 380, 381)*
12a. Ethanolamine phosphate	CTP	CDP-ethanolamine^{j}	*(256)*

GROUP II.  $\mathrm{R{-}COOH + ATP \rightleftarrows R{-}\overset{\overset{O}{\|}}{C}{-}O{-}\underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{P}}{-}adenosine + PP_i}$	References
1. Formic acid[l]	(*382*; cf. ref. *89*)
2. Acetic acid[m]	(*292*, *383–390*)
3. Propionic acid[m]	(*51*)
4. Butyric acid[j]	(*391*)
5. Various fatty acids[j]	(*52*)
6. C_5-C_{22} straight saturated acids[j]	(*392*)
7. Octanoic acid[j]	(*393*)
8. Oleic, linoleic, linolenic, and chaulmoogric acids[j]	(*392*)
9. Benzoic acid and related acids[j]	(*394–396*)
10. Cholic acid[n]	(*397*; cf. ref. *89*)
11. Pantoic acid	(*398*, *399*)
12. Carbonic acid[o]	(*400–402*)
13. Amino acids (all common amino acids which occur in proteins)[p]	(*110*, *403–411*)

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[a] Found in yeast, and nucleated vertebrate and invertebrate cells such as liver, chicken erythrocytes, tumors, mammary gland, spleen, sea urchin, and meal worm (Atkinson and Morton, unpublished work).

[b] Present in human red cells, yeast, and liver.

[c] Present in rat, pig, and pigeon liver.

[d] Found in liver and yeast.

[e] In yeast and plants.

[f] Found in yeast, rat liver, muscle, and plants.

[g] Found in yeast.

[gg] Found in plants.

[h] Found in rat liver.

[i] Found in *Lactobacillus arabinosus.*

[j] Found in mammalian liver.

[k] Found in rat seminal vesicle.

[l] Found in *Lactobacillus casei.*

[m] In pig and rabbit heart muscle, and yeast.

[n] Found in guinea pig liver.

[o] Found in yeast, heart muscle, and bacteria.

[p] Widely distributed in animals, plants, and microorganisms.

TABLE VIII (*Continued*)

GROUP III. $>NH$	$-\overset{\mid}{\underset{\mid}{C}}-O-\overset{O}{\overset{\Vert}{\underset{\underset{OH}{\mid}}{P}}}-O-\overset{O}{\overset{\Vert}{\underset{\underset{OH}{\mid}}{P}}}-OH$	$>N-\overset{\mid}{\underset{\mid}{C}}-$	References
1. Glutamine	PRPP	Glutamyl ribotide[q]	(*412–414*)
2. Anthranilic acid	PRPP	*N*-5′-Phosphoribosyl anthranilate[q,r]	(*415*, *416*)
3. Nicotinic acid	PRPP	Nicotinic acid nucleotide[s]	(*367*, *367a,b*, *368*)
4. Nicotinamide	PRPP	Nicotinamide nucleotide[t]	(*417*, *418*)
5. Orotic acid	PRPP	Orotidine-5′-phosphate[u]	(*77*, *419*, *420*)
6. Uracil	PRPP	UMP[u]	(*165*, *322*, *420*)
7. Adenine	PRPP	AMP[d]	(*78*, *79*, *88*, *89*, *166*, *417*, *421–423*)
8. Hypoxanthine	PRPP	IMP[d,v]	(*79*, *285*, *417*, *424*, *425*)
9. Guanine	PRPP	GMP[d,v]	(*79*, *417*)
10. 6-Mercaptopurine	PRPP	Mercaptopurine ribotide[w]	(*426*)
11. Azaguanine	PRPP	Azaguanosine-5′-phosphate[q]	(*246*)
12. 4-Aminoimidazole-5-carboxamide	PRPP	4-Aminoimidazole-5-carboxamide ribotide[j]	(*78*, *426*)

GROUP IV.	PYROPHOSPHOROLYSIS OF POLYDEOXYRIBONUCLEOTIDE[x]		References
(Polydeoxyribonucleotide)$_n$	Deoxy-ATP + Deoxy-GTP + Deoxy-CTP + TTP	(Polydeoxyribonucleotide)$_{n+4}$	(*89*, *166*, *167*, *223*, *427*)

GROUP V.	PYROPHOSPHOROLYSIS OF MIXED INORGANIC ANHYDRIDES		References
1. Hydrochloric acid	ATP	Adenylchloride	*(89)*
2. Nitric acid	ATP	Adenylnitrate	*(89)*
3. Selenic acid	ATP	Adenylselenate[g]	*(179, 428)*
4. Sulfate	ATP	Adenylsulfate[d]	*(177–179, 428–431)*
5. Sulfurous acid	ATP	Adenylsulfite [g]	*(179)*
6. Chromic acid	ATP	Adenylchromate[g]	*(179)*
7. Molybdic acid	ATP	Adenylmolybdate[g]	*(179)*
8. Tungstic acid	ATP	Adenyltungstate[g]	*(179)*

GROUP VI.	MISCELLANEOUS PYROPHOSPHORYLYSES		References
1. Phenol	ATP	Adenylphenol	*(89)*
2. Citrulline	ATP	*O*-Adenylcitrulline[h]	*(89)*
3. Aspartic acid	ATP	*N*-Adenylaspartate[h]	*(89)*

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[q] Found in pigeon liver.

[qq] *N*-5′-Phosphoribosyl anthranilate is a probable intermediate in formation of indole-3-glycerol phosphate; similarly glutamyl ribotide is a probable intermediate in formation of 5-phosphoribosylamine.

[r] Found in *E. coli*.

[s] Present in human red cells, yeast, and liver.

[t] Found in red cells and liver.

[u] Found in *Lactobacillus* spp. but also occurs in animal tissues.

[v] Found in pigeon liver, human red cells, and microorganisms.

[w] Found in beef liver.

[x] Occurs in *E. coli* and liver.

these processes ensures that synthesis may proceed continuously. Moreover, there are numerous hydrolytic reactions which degrade the essential polymers, and resynthesis is also dependent on activation by ATP. Hence this reactive phosphate is required not only for growth processes but also for maintenance of the living cell.

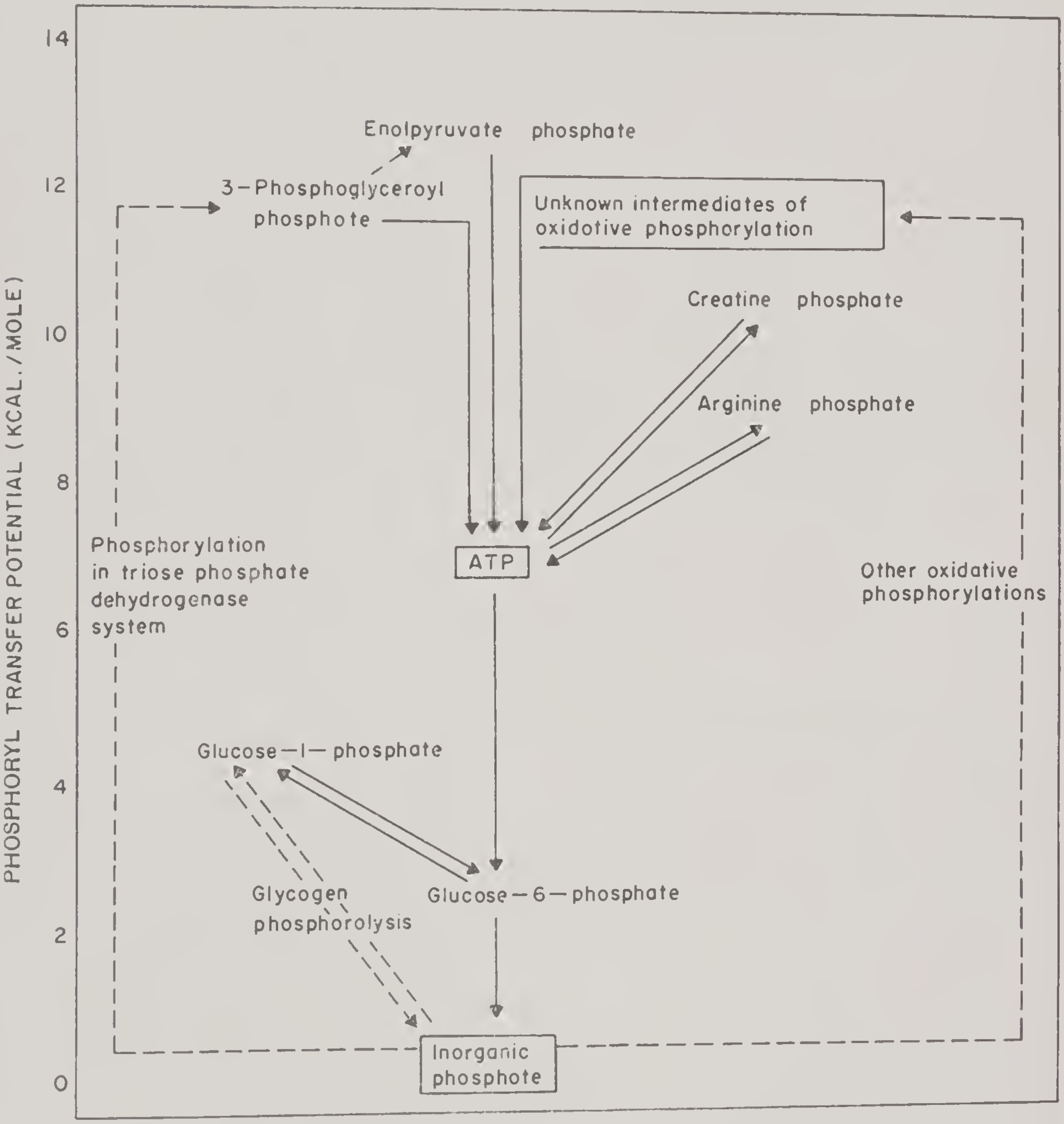
PHOSPHORYL TRANSFER POTENTIAL (KCAL./MOLE)
14
12
10
8
6
4
2
0
Enolpyruvate phosphate
3-Phosphoglyceroyl phosphate
Unknown intermediates of oxidative phosphorylation
Creatine phosphate
Arginine phosphate
ATP
Phosphorylation in triose phosphate dehydrogenase system
Other oxidative phosphorylations
Glucose-1-phosphate
Glycogen phosphorolysis
Glucose-6-phosphate
Inorganic phosphate

FIG. 2. Some free-energy changes in the biosynthesis of phosphates.

The phosphoryl transfer potential of ATP is sufficiently high to ensure phosphorylation, and thus activation, of numerous metabolites such as glucose. However, it is low enough to permit ready phosphorylation of ADP to ATP by phosphoryl transfer from compounds of high transfer potential such as enolpyruvate phosphate and 3-phosphoglyceroyl phosphate. The phosphagens (e.g., creatine phosphate and arginine phosphate) like ATP, are of intermediate transfer potential, and transfer of phosphoryl groups between ATP and these compounds occurs freely. The free-energy changes of some of these reactions are shown in Fig. 2. The central position of ATP is evident.

All the processes discussed above are reversible, but some of the free-energy changes are so large that in normal physiological conditions they proceed effectively in only one direction. This results in a definite sequence of reactions during phosphate biosynthesis.

A. Synthesis of ATP from Inorganic Phosphate

The Embden-Meyerhof-Parnas pathway of hexose phosphate utilization has been demonstrated in many living organisms. In this process 3-phosphoglyceraldehyde is converted by an oxidative process into a 3-phosphoglyceroyl thioester. Phosphorolysis of this compound (Table VII) results in the formation of 3-phosphoglyceroyl phosphate with uptake of inorganic phosphate. In this way the phosphoryl group of inorganic phosphate is raised from zero transfer potential to that of the acyl phosphate (11.8 kcal./mole).* Phosphoryl transfer to ADP catalyzed by phosphoglyceric kinase (Table III) leads to ATP formation. Since synthesis of a high potential phosphate by phosphorolysis would not have been possible without prior oxidative formation of the thioester, this activation of inorganic phosphate is an example of oxidative phosphorylation (cf. Vol. I, Chapter 10).

Among anaerobic microorganisms oxidative decarboxylation of pyruvate is coupled with phosphorylation of ADP to ATP. Acetyl coenzyme A, an intermediate in the process, undergoes phosphorolysis to acetyl phosphate (Table VII). This compound, which has a high phosphoryl transfer potential (10.1 kcal./mole)* phosphorylates ADP in the presence of acetokinase (Table III). Acetic acid formed in this reaction is frequently an end product of metabolism (*440*). In higher plants and animals this phosphorolysis has not been demonstrated, but instead acetyl coenzyme A is converted into citrate by condensation with oxaloacetate (*441*). A further phosphorylation occurs during decarboxylation of α-ketoglutarate which is formed from citrate. Phosphorolysis of the intermediate, succinyl coenzyme A, results in uptake of inorganic phosphate and formation of succinate and probably of coenzyme A phosphate (Table VII). In animal systems it seems likely that guanosine triphosphate is first formed by phosphoryl transfer to guanosine diphosphate (*125*). ATP is then formed by the reaction catalyzed by nucleoside diphosphokinase (Table III). In plant systems the requirement for guanosine nucleotides in this reaction has not been demonstrated (*335, 336*).

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* See Table II.

Much more ATP is formed by oxidative phosphorylation associated with electron transfer from substrates to terminal oxidants (*90, 442, 443*). This process involves pyridine nucleotides, flavoproteins, cytochromes, and possibly other compounds capable of reversible oxidation. In animals and higher plants oxygen itself is the terminal electron acceptor. In microorganisms sulfate, nitrate, fumarate, and probably other compounds have a similar function (*444, 445*). In photosynthetic phosphorylation in green plants a product of photolysis of water is the terminal oxidant (*446*). Despite the diversity of electron donors, acceptors, and carriers, the final product of oxidative phosphorylation in all these living systems is ATP. The mechanism is still obscure, but phosphorolysis of an intermediate which has been activated by oxidation* and subsequent phosphoryl transfer to ADP seems likely. Though the importance of other nucleoside triphosphates is increasingly apparent, their formation largely depends on a supply of ATP. Undoubtedly the ADP-ATP system dominates phosphoryl transfer reactions.

B. ACTIVATION OF METABOLITES

In general nutrients are absorbed as unphosphorylated compounds of low molecular weight. Within the animal or plant they are usually transported in this form (e.g., glucose in the animal or sucrose in the plant). Before these compounds can be oxidized for provision of energy they are converted into activated derivatives. Oxidation of monosaccharides almost always involves preliminary phosphorylation by ATP in the presence of suitable kinases, although there are bacterial systems (*440*) which can oxidize unphosphorylated carbohydrates. Transfer of a phosphoryl group from ATP (transfer potential, 7 kcal./mole)† to glucose, with formation of glucose-6-phosphate (transfer potential, 3.3 kcal./mole)† is a typical carbohydrate activation. During subsequent oxidative degradation of glucose-6-phosphate, inorganic phosphate is raised to the high transfer potential of 3-phosphoglyceroyl phosphate, as discussed above, and the phosphoryl group provided by ATP in the original activation is raised to the even higher transfer potential (12.8 kcal./mole)† of enolpyruvate phosphate. This compound readily phosphorylates ADP (Table III) to form ATP and thus replenish the pool of reactive phosphoryl groups, as illustrated in Fig. 2.

By the mutase reaction (Table V) glucose-6-phosphate is converted into glucose-1-phosphate. This activated carbohydrate can be converted into the storage polysaccharide glycogen, with little free energy change, by reversal of phosphorolysis‡ (see Table I). Phosphate is returned to the inorganic phosphate pool, from which it can be regained in a reactive form by the forward reaction of glycogen phosphorylase (Table VII). The extent of polysaccharide formation and the nature of the products differs considerably among living organisms (*108a, 307*).

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* Alternatively the organic phosphate may be formed prior to activation of the phosphoryl group by oxidation.

† See Table II.

In multicellular organisms carbohydrate is often transported between tissues. This usually involves dephosphorylation by phosphatases and subsequent reactivation by kinases (*447*).

Activation of fatty acids, derived either from fats or proteins requires adenyl transfer from ATP (Table VIII) before they are converted into coenzyme A derivatives and subsequently oxidized. In this way ATP is converted into AMP and inorganic pyrophosphate. The AMP is converted into ADP by phosphoryl transfer from ATP (adenylate kinase reaction; Table III) and the pyrophosphate is converted into orthophosphate by pyrophosphatase (*432, 433*). Regeneration of ATP from these products can take place by oxidative phosphorylation as discussed above.

C. Maintenance of the Reactive Phosphoryl Pool

In a uniform environment, the formation and breakdown of ATP by the processes discussed above would occur at uniform rates, with maintenance of adequate levels of ATP, ADP, and inorganic phosphate. However, a sudden alteration of requirement for phosphoryl groups would lower the ATP level. Alternatively, depletion of phosphoryl acceptors would result from excessive formation of compounds with very high transfer potentials (e.g., enolpyruvate phosphate). In either case there could be marked physiological disturbances.

In order to maintain an optimum balance between ATP, ADP, and inorganic phosphate, particularly in response to changes of external environment, a reserve pool of reactive phosphoryl groups is required. Just as buffer systems in the cell maintain an optimum hydrogen ion concentration, interaction between the phosphoryl reserve and the ATP-ADP system would buffer the latter at an optimum level of phosphoryl groups.

The phosphagens serve this purpose, since the transfer potentials of these compounds are close to that of ATP (Fig. 2) (see Table II). The free-energy change of phosphoryl transfer between the ATP-ADP system and the phosphagen is probably small under physiological conditions. As shown in Table I, this is particularly dependent on pH and magnesium concentration. The effect of magnesium ion on the hexokinase system, for example, is much smaller than on systems involving phosphagen (see Table I).

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‡ Polysaccharides are also formed from hexose phosphates by alternative pathways involving uridine nucleotides (*108b,c*).

The continual transfer of phosphoryl groups from ATP to creatine, and from creatine phosphate to ADP in skeletal muscle is evident from the finding of identical turnover of radioactive phosphate in the reactive phosphoryl groups of creatine phosphate and ATP (*448*). A sudden loss of ATP during muscular action cannot be immediately restored by oxidative phosphorylation. However, phosphoryl transfer from creatine phosphate immediately buffers the ATP-ADP system at a suitable level.

If the main function of the phosphagens is to buffer the ATP-ADP system, it would be expected that the most effective buffering (both in amount of phosphagen and activity of kinase) would be found in those tissues subject to the greatest variation of physiological demands or environmental conditions. The large requirement for ATP in working skeletal muscle is in marked contrast to the relatively small requirement for this compound when the same muscle is at rest. Probably no other tissue is subject to such wide variations in the demand for reactive phosphoryl groups. It is not surprising, therefore, that the highest levels of creatine phosphate and of creatine kinase are found in vertebrate skeletal muscle (*104*). The involuntary muscles, particularly the heart muscles, work at a more uniform rate, and can ensure an adequate phosphoryl supply with smaller phosphagen reserves. Other organs such as the liver are subject to even smaller environmental fluctuations, and have very small phosphagen reserves (*104*).

Maintenance of the pool of reactive phosphoryl groups against changes induced by variations in the external environment is probably the main function of phosphagens. It would therefore be expected that the nature and amount of phosphagens and related kinases would show more variation on a phylogenetic basis than the nature and amount of compounds and enzymes involved in the essential reactions of oxidative phosphorylation and substrate activation. Only the creatine-creatine phosphate system has been found to have phosphagen properties in vertebrates. The invertebrates, which are subject to greater environmental change than most vertebrates, contain in addition a number of other guanidine-type phosphagens. The best characterized of these are the phosphoryl derivatives of arginine, glycocyamine, taurocyamine, and lombricine (*193, 449, 450, 451*) (cf. Table III, Group VII).

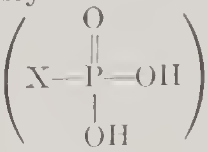
The term phosphagen has previously been used for phosphorylated guanidines. The presence of these compounds has not been detected in plants and microorganisms (*450, 451*). If the term is extended to include all compounds which buffer the phosphoryl level of the ATP-ADP system it would include metaphosphate and possibly phosphorylated RNA. As shown in Table III there are kinases which catalyze phosphoryl transfer from these compounds to ADP, and it seems likely that the free-energy changes of these reactions are small. Metaphosphate is found in relatively large amounts in yeast and certain bacteria. The level of metaphosphate changes considerably in different metabolic phases (e.g., spore formation) (*206, 452*).

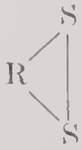
It has recently been claimed that RNA from yeast or pancreas could be phosphorylated by ATP in the presence of muscle, liver, or yeast extracts (*58, 183, 224, 225*).

In order that the level of phosphorylation of the ATP-ADP system should respond to physiological stimuli (e.g., hormones) the absence of a phosphoryl buffering system may be necessary. This may be especially the case in certain organs or at definite stages of differentiation and development of living systems. The large changes in the balance of the ATP-ADP-inorganic phosphate system during maturation of fruits (*453*) probably implies the absence of an effective phosphoryl buffering system. In view of the probable high phosphoryl transfer potential of phytate (see Section II, C, 5), this and related inositol phosphates may be important phosphogens of certain germinating plant seeds.

An alternative mechanism by which activated phosphoryl groups of such compounds as acyl phosphates can be returned to the inorganic phosphate pool, when there is a shortage of ADP, is by hydrolysis in the presence of specific phosphatases (*265, 454, 455*). The activity of these phosphatases may be under hormonal or other physiological control (*455, 456*). Adenosine triphosphatases (*457, 458*) clearly may have the same function, and permit continuous oxidation of metabolites without a corresponding increase in the reactive phosphoryl pool (*262, 266*).

The complex interrelationships of phosphate biosynthesis are understandable in terms of the free-energy changes involved. It is characteristic of living systems, which must function in a restricted range of temperature and hydrogen ion concentration, that most important reactions are dependent on a supply of adenyl or phosphoryl groups from adenosine triphosphate. Equally essential is the availability of phosphoryl groups at their lowest potential as inorganic phosphate. Elucidation of the processes by which phosphoryl groups are activated and by which adenosine triphosphate is used has been one of the major achievements of modern biochemistry. Though a century has passed since Liebig (*1*) drew attention to the need for phosphate in living things, it is not twenty years since Lipmann (*81*) pointed out the metabolic significance of the graded transfer potentials of phosphorylated metabolites. It is a measure of the rapid progress of biochemistry that such a complete picture of the reactions involved in phosphate biosynthesis is now available. This picture is derived from studies on an extremely limited range of biological material. Combination of such information has proved extremely valuable in providing a guide for further work at the comparative level. It may, however, lead to gross oversimplification and to serious errors. Only by detailed examination of the reactions in a given species can the validity of present concepts of phosphate biosynthesis be established. This is the outstanding task now facing the comparative biochemist.

P_i	Inorganic orthophosphate
PP_i	Inorganic pyrophosphate
X—P	Phosphoryl derivatives  $\left(X-\underset{\underset{\displaystyle OH}{\mid}}{\overset{\overset{\displaystyle O}{\Vert}}{P}}-OH \right)$
AMP	Adenosine-5′-monophosphate
ADP	Adenosine-5′-diphosphate
ATP	Adenosine-5′-triphosphate
GMP	Guanosine-5′-monophosphate
GDP	Guanosine-5′-diphosphate
GTP	Guanosine-5′-triphosphate
CMP	Cytidine-5′-monophosphate
CDP	Cytidine-5′-diphosphate
CTP	Cytidine-5′-triphosphate
UMP	Uridine-5′-monophosphate
UDP	Uridine-5′-diphosphate
UTP	Uridine-5′-triphosphate
deoxy-AMP	Deoxyadenosine-5′-monophosphate
deoxy-ADP	Deoxyadenosine-5′-diphosphate
deoxy-ATP	Deoxyadenosine-5′-triphosphate
deoxy-GMP	Deoxyguanosine-5′-monophosphate
deoxy-GDP	Deoxyguanosine-5′-diphosphate
deoxy-GTP	Deoxyguanosine-5′-triphosphate
deoxy-CMP	Deoxycytidine-5′-monophosphate
deoxy-CDP	Deoxycytidine-5′-diphosphate
deoxy-CTP	Deoxycytidine-5′-triphosphate

TMP	Thymidine-5′-monophosphate
TDP	Thymidine-5′-diphosphate
TTP	Thymidine-5′-triphosphate
IMP	Inosine-5′-monophosphate
IDP	Inosine-5′-diphosphate
ITP	Inosine-5′-triphosphate
$[a_B]$	Activity of reactant *B*
(X)	Molar concentration of X
coenzyme A-SH or CoA	Unsubstituted coenzyme A
coenzyme A-S-P	*S*-Phosphoryl coenzyme A
succinyl-S-CoA	*S*-Succinyl coenzyme A
acetoacetyl-S-CoA	*S*-Acetoacetyl coenzyme A
enzyme-P	Phosphorylated enzyme
DPN	Diphosphopyridine nucleotide
NMN	Nicotinamide riboside-5′-phosphate
PRPP	5-Phosphoribosyl-1-pyrophosphate
CDP-choline	Cytidine-5′-diphosphocholine
CDP-ethanolamine	Cytidine-5′-diphosphoethanolamine
CDP-ribitol	Cytidine-5′-diphosphoribitol
cytidine-2′,3′-P	Cytidine-cyclic-2′,3′-phosphoester
uridine-2′,3′-P	Uridine-cyclic-2′,3′-phosphoester
RNA	Ribonucleic acid
RNA "core"	Undialyzable residue after digestion of RNA with ribonuclease
$R(SH)_2$, 	Reduced and oxidized forms of 1,3-propanedithiol

Substrate-enzyme complexes are shown thus:

glucosyl-(sucrose phosphorylase)
3-phosphoglyceroyl-*S*-(phosphoglyceraldehyde dehydrogenase)
β-aspartyl-*S*-(aspartaldehyde dehydrogenase)

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CHAPTER 2

Utilization of Free Energy for the Biosynthesis of Saccharides

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I. Introduction

Carbohydrates have the dual role of fuels and of structural elements in the organism. Their role as fuels can be taken as a good example of unity in biochemical organization. Thus, as far as we know, glucose and glycogen or starch are present in every organism and are used as fuels. Although there are more pathways for carbohydrate dissimilation than was suspected some years ago, many of the reactions are common to numerous groups of organisms. On the other hand, the examination of the structural carbohydrates reveals an astonishing number of characteristic polysaccharides, so that such a study can be taken as an example of variety in biochemical organization.

The energy of photosynthetic, oxidative, or anaerobic reactions is used in the cells to form hexose phosphates from lower molecular weight compounds. These hexose phosphates may then be modified by a series of interconversion reactions. The chains can be elongated or shortened, aldoses can be transformed into ketoses, uronic acids, or polyols, and phosphate or amino groups can be added or removed. At present it seems impossible to integrate all the knowledge we have or to give a sketch of the master plan, if it is possible to draw one. Some of these reactions lead to the formation of monosaccharides substituted at the reducing group. These glycosyl derivatives may then react with acceptors and lead to the synthesis of polysaccharides.

The bulk of cell sugars is in the form of polysaccharides, either reserve polysaccharides such as glycogen, starch, or fructans, or structural elements such as cellulose and hemicellulose in plants. The reader is referred to specialized books (*1, 2*) and Volumes III and IV of this treatise for a description of the enormous variety of polysaccharides present in nature.

II. The Synthesis of Monosaccharides

A. Synthesis from CO_2

Studies in photosynthetic organisms have shown that the fixation of CO_2 during photosynthesis occurs by the reaction

$$\text{Ribulose diphosphate} + CO_2 \rightleftharpoons 2\ \text{Phosphoglyceric acid} \tag{1}$$

Phosphoglyceric acid is then reduced to triose phosphate. Ribulose diphosphate can be regenerated by a series of reactions involving pentose, hexose, and heptulose phosphates, so that the whole process becomes cyclic and a net synthesis of monosaccharides takes place.

B. The Reversion of Glycolysis: Pyruvic Acid to Glucose

The synthesis of glycogen from glucose is an endergonic process, with $\Delta F^\circ = +4000$ cal. approximately. The sequence of reactions (2–5) which lead from glucose to glycogen *in vivo* is as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP} \tag{2}$$

$$\text{Glucose-6-phosphate} \xrightarrow{\text{Phosphoglucomutase}} \text{Glucose-1-phosphate} \tag{3}$$

$$\text{Glucose-1-phosphate} \xrightarrow{\text{Phosphorylase}} \text{Glycogen} + P_i \tag{4}$$

$$\textit{Sum:}\quad \text{Glucose} + \text{ATP} \xrightarrow{\qquad\qquad} \text{Glycogen} + \text{ADP} + P_i \tag{5}$$

The formation of glycogen from glucose is thus coupled with the hydrolysis of ATP,[*] an exergonic reaction with $\Delta F^\circ = -7700$ cal.,[†] and therefore the ΔF° of the total process is about -3700 cal.

If, however, the synthesis of glycogen is mediated by the newly

[*] Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; UDPG, uridine diphosphate glucose; DPN, diphosphopyridine nucleotide; $DPNH_2$, reduced form of DPN; TPN, triphosphopyridine

discovered enzyme UDPG-glycogen transglucosylase (*3a*), the probable reaction sequence would be as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose-6-phosphate} + \text{ADP} \qquad (5a)$$

$$\text{Glucose-6-phosphate} \xrightarrow{\text{Phosphoglucomutase}} \text{Glucose-1-phosphate} \qquad (5b)$$

$$\text{UDP} + \text{ATP} \xrightarrow{\text{Nucleotide diphosphate kinase}} \text{UTP} + \text{ADP} \qquad (5c)$$

$$\text{UTP} + \text{Glucose-1-phosphate} \xrightarrow{\text{UDPG-pyrophosphorylase}} \text{UDPG} + \text{PP}_i \qquad (5d)$$

$$\text{UDPG} \xrightarrow{\text{UDPG-glycogen transglucosylase}} \text{Glycogen} + \text{UDP} \qquad (5e)$$

Sum: $$\text{Glucose} + 2\ \text{ATP} \rightarrow \text{Glycogen} + 2\ \text{ADP} + \text{PP}_i \qquad (5f)$$

Since the free energy of hydrolysis of inorganic pyrophosphate is considered to be about equal to that of the terminal phosphate of ATP, the total free energy change would be almost the same for either of the two pathways. If, on the other hand, pyrophosphate is degraded to inorganic phosphate the total $\Delta F°$ would be about −11,700 cal. More energy would be expended through the UDPG pathway, but the equilibrium would be more displaced toward glycogen synthesis.

The formation of carbohydrate from lactate affords another example of the coupling with exergonic reactions.

It has long been known that lactate or pyruvate can be converted, especially in liver, to glucose, and it was assumed that this occurred through a reversal of glycolysis. It is apparent, however, that the equilibrium for the over-all transformation is very unfavorable, as shown by Reactions 6 and 7, which represent the sum of the well known glycolytic reactions in the backward direction.

$$2\ \text{Lactic acid} + 3\ \text{ATP} + 2\ \text{H}_2\text{O} \rightarrow \text{Glycogen} + 3\ \text{ADP} + 3\ \text{P}_i\ (\Delta F° = +28{,}400\ \text{cal.}) \qquad (6)$$

$$2\ \text{Lactic acid} + 2\ \text{ATP} + 2\ \text{H}_2\text{O} \rightarrow \text{Glucose} + 2\ \text{ADP} + 2\ \text{P}_i\ (\Delta F° = +32{,}000\ \text{cal.}) \qquad (7)$$

The positive value of $\Delta F°$ is mainly due to Reactions 8–10, which in glycolysis take place towards the left.

$$\text{Glucose-6-phosphate} + \text{ADP} \xrightarrow{\text{Hexokinase}} \text{Glucose} + \text{ATP} \qquad (8)$$

$$\text{Fructose-1,6-diphosphate} + \text{ADP} \xrightarrow{\text{Phosphofructokinase}} \text{Fructose-6-phosphate} + \text{ATP} \qquad (9)$$

$$\text{Pyruvate} + \text{ATP} \xrightarrow{\text{Pyruvate kinase}} \text{Phosphopyruvate} + \text{ADP} \qquad (10)$$

---

nucleotide; TPNH_2, reduced form of TPN; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; $\Delta F°$, standard free energy change; $\Delta F_H°$, standard free energy of hydrolysis.

† This new value for ATP (*3*) has been used to correct the ΔF's given by Krebs and Kornberg (*4*) for the glycolytic reactions.

Each one of these reactions has a positive ΔF° of 5000–6000 cal. It should be noted that Reaction 10 must take place twice for each molecule of glucose synthesized. Reactions 8 and 9 could be driven from left to right by a high ratio of ADP to ATP, but the opposite situation is needed for Reaction 10 to proceed. It is difficult to visualize how both conditions could be met within the same cell, although the reactions might take place at different sites. Krebs and Kornberg have suggested (*4*) that special by-passes are utilized in order to effect a net synthesis of glucose and glycogen. Fructose-1,6-diphosphate and glucose-6-phosphate would be dephosphorylated through the agency of the two specific phosphatases (*5, 6*).

Reactions 8 and 9 would be replaced by Reactions 11 and 12.

$$\text{Fructose-1,6-diphosphate} \rightarrow \text{Fructose-6-phosphate} + \text{P}_i \tag{11}$$

$$\text{Glucose-6-phosphate} \rightarrow \text{Glucose} + \text{P}_i \tag{12}$$

Instead of Reactions 6 and 7, the over-all reactions would be:

$$2\ \text{Lactic acid} + 4\ \text{ATP} + 3\ H_2O \rightarrow \text{Glycogen} + 4\ \text{ADP} + 4\ \text{P}_i\ (\Delta F^\circ \rightarrow +20{,}700\ \text{cal.}) \tag{13}$$

$$2\ \text{Lactic acid} + 4\ \text{ATP} + 3\ H_2O \rightarrow \text{Glucose} + 4\ \text{ADP} + 4\ \text{P}_i\ (\Delta F^\circ \rightarrow +16{,}600\ \text{cal.}) \tag{14}$$

For the phosphorylation of pyruvate (Reaction 10), Krebs and Kornberg (*4*) propose a more complex mechanism (Reactions 15–18).

$$\text{Pyruvate} + \text{TPNH}_2 + CO_2 \xrightarrow{\text{"Malic enzyme"}} \text{Malate} + \text{TPN} \tag{15}$$

$$\text{Malate} + \text{DPN} \xrightarrow{\text{Malic dehydrogenase}} \text{Oxalacetate} + \text{DPNH}_2 \tag{16}$$

$$\text{Oxalacetate} + \text{ITP} \longrightarrow \text{Phosphopyruvate} + CO_2 + \text{IDP} \tag{17}$$

$$\textit{Sum:}\ \text{Pyruvate} + \text{TPNH}_2 + \text{DPN} + \text{ITP} \longrightarrow \text{Phosphopyruvate} + \text{DPNH}_2 + \text{TPN} + \text{IDP} \tag{18}$$

Apparently, nothing would be gained by this "detour," since the free energy of hydrolysis of ITP is similar to that of ATP and the oxidation reduction potentials of the DPN and TPN systems are practically identical (so that the corresponding ΔF° would cancel out). However, as suggested by Krebs, this pathway affords a possibility of linking the phosphorylation of pyruvate with the following reactions that can drive Reaction 18 from left to right: (*a*) TPNH_2 might be supplied partly by the oxidation of glucose-6-phosphate and partly by a reversal of oxidative phosphorylation, according to the reaction:

$$\text{Reduced flavoprotein} + \text{TPN} + \text{ATP} \rightarrow \text{Flavoprotein} + \text{TPNH}_2 + \text{ADP} + \text{P}_i \tag{19}$$

(*b*) $DPNH_2$ would be reoxidized by the flavoprotein-cytochrome-O_2 system; (*c*) ITP would be produced from ATP by the enzyme nucleotide diphosphate kinase:

$$ATP + IDP \rightleftarrows ADP + ITP \qquad (20)$$

Figure 1 shows the complete pathway from lactic acid to glucose and glycogen. Two important results support this formulation. One is the fact that when labeled CO_2 is incorporated into glucose in liver, the label appears in carbons 3 and 4 of the glucose, as required by the reaction sequence presented (7). The other is the formation of glucose equally labeled in carbons 1, 2, 5 and 6, when either α- or β-C^{14}-labeled pyruvate or lactate are fed to the animal (8, 9). Therefore, the α- and

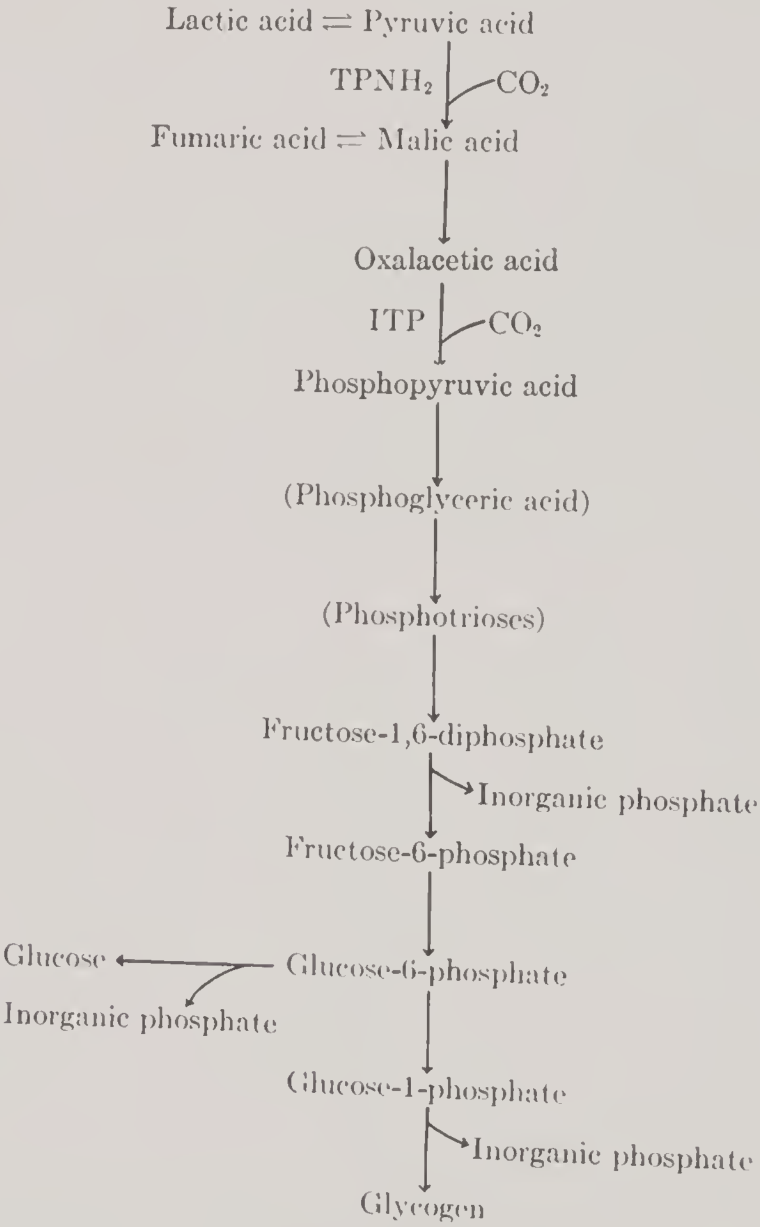


FIG. 1. Synthesis of glycogen from lactic acid.

β-carbon must be equivalent at some stage. This could not occur in a simple reversal of glycolysis, but it would be readily explained by the pathway shown in Fig. 1 if it is further assumed that malic acid is rapidly equilibrated with the symmetrical compound, fumaric acid.

Similar experiments, performed with labeled pyruvate, point however to a different mechanism in muscle (*9a*).

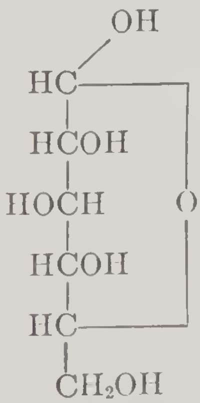
Pyruvate can be considered as a key metabolite. The transformation of amino acids and fats to carbohydrates takes place through the intermediary formation of pyruvic acid.

C. INTERCONVERSION OF SUGARS (*10–17*)

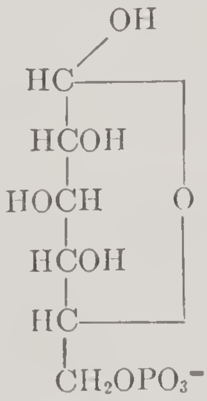
1. *General Mechanisms*

The sugars formed by photosynthesis, by reversal of glycolysis, or by any other mechanism may be converted into other sugars. Although certain reactions take place on the free sugars, in most cases certain derivatives are first formed.

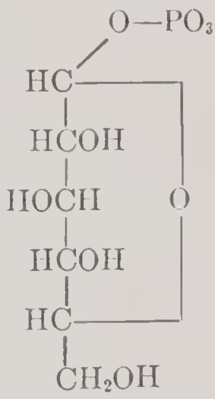
a. The Formation of Derivatives. The most commonly found derivatives of sugars are the sugar phosphates. A typical reaction which leads to their formation is that catalyzed by hexokinase, in which a phosphate group from ATP is transferred to glucose (I) to give glucose-6-phosphate (II).



α-Glucose
(pyranose form)
(I)



α-Glucose-6-phosphate
(pyranose form)
(II)



α-Glucopyranose-1-phosphate
(III)

Enzymes present in most organisms catalyze this reaction as well as the phosphorylation of fructose and mannose to the 6-phosphates. These and other reactions which have been studied are shown in Table I.

In most cases the phosphate group is introduced in a primary alcohol group, that is, at position 6 of aldoses, position 5 of pentoses, positions 1 or 6 of ketohexoses, and positions 1 or 5 of ketopentoses. Galactose and the corresponding hexosamine are exceptions to this rule be-

TABLE I

KINASES

General reaction: ATP + Sugar → Sugar phosphate + ADP

Substrate	Product	Origin	Reference
D-Glucose	D-Glucose-6-phosphate	Widespread	*18*
D-Mannose	D-Mannose-6-phosphate	Widespread	*18*
D-Fructose	D-Fructose-6-phosphate	Widespread	*13*
D-Fructose	D-Fructose-1-phosphate	Muscle, liver	*11, 13*
D-Galactose	D-Galactose-1-phosphate	Yeast, liver, brain	*19, 20*
D-Fructose-6-phosphate	D-Fructose-1,6-disphosphate	Widespread (yeast, muscle)	*13*
D-Ribose	D-Ribose-5-phosphate	Liver	*21*
L-Ribulose	L-Ribulose-5-phosphate	*Lactobacillus, Aerobacter*	*22, 23, 23a,b*
D-Xylulose	D-Xylulose-5-phosphate	*Lactobacillus*, Liver	*24, 24a*
D-2-Deoxyribose	D-2-Deoxyribose-5-phosphate(?)	Liver, *Escherichia coli*	*21, 24b*
D-Ribose-5-phosphate	D-Ribose-1,5-diphosphate	Liver	*25*
D-Ribulose-5-phosphate	D-Ribulose-1,5-diphosphate	Spinach	*26*
D-Glucosamine	D-Glucosamine-6-phosphate	Brain, yeast, *Neurospora crassa, Schistosoma mansoni*	*27, 28, 29, 30*
D-Galactosamine	D-Galactosamine-1-phosphate	Liver and yeast	*20*
D-Acetylglucosamine	D-Acetylglucosamine-6-phosphate	Animal tissues, *Escherichia coli*	*31, 32*
D-Acetylgalactosamine	D-Acetylgalactosamine-1-phosphate	Animal tissues	*32*
D-Gluconate	D-6-Phosphogluconate	*Escherichia coli*	*33*

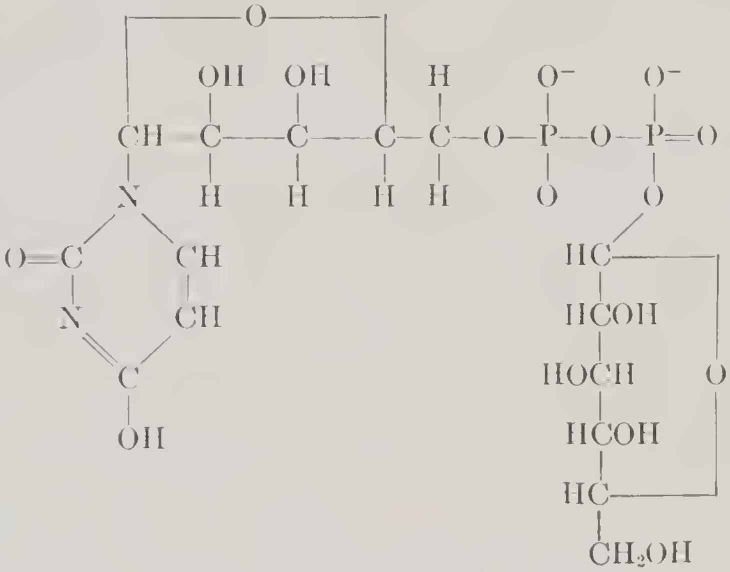
cause they are phosphorylated at position 1, forming an ester-glycoside bond.

A special case of these phosphorylation reactions is the transfer of a pyrophosphate group (*34, 34a*) as follows:

$$\text{ATP} + \text{Ribose-5-phosphate} \rightarrow \text{AMP} + \text{1 Pyrophospho-5-phosphoribose} \qquad (21)$$

This is the only case known at present, but this type of reaction might well be more general. The reaction product is involved in the biosynthesis of nucleotides as a donor of the ribose-5-phosphate group.

Another type of derivative commonly formed is the uridine diphosphate-sugar compound. The best known is UDPG (IV). UDP derivatives of the following sugars have been isolated from natural sources: glucose (*35*), galactose (*36*), acetylglucosamine (*37*), acetylgalactosamine (*38*), acetylglucosamine-6-phosphate (*39*), galactosamine sulfate (*39*), xylose (*40*), L-arabinose (*40*), rhamnose (*40a*), glucuronic acid (*41*), galacturonic acid (*42*), polyacetylneuraminic acid (*42a*), and muramic acid (*43*).



Uridine diphosphate glucose (UDPG)

(IV)

The synthesis of UDP-sugar compounds takes place as follows:

$$\text{UTP} + \text{glycosyl-1-phosphate} \rightleftharpoons \text{UDP-glycosyl} + \text{PP}_i \qquad (22)$$

Enzymes catalyzing such a reaction with the 1-phosphates of glucose (*44, 44a,b,c*), galactose (*45, 45a*), acetylglucosamine (*46*), D-xylose (*47*), L-arabinose (*47*) and glucuronic and galacturonic acid (*47a*), have been described, as well as a similar one (*48*) in which guanosine triphosphate (GTP) and mannose-1-phosphate give guanosine diphosphate mannose (*49*).

Reaction 22 has an equilibrium constant of about one in the case of the corresponding glucose derivative, so that such a reaction is not very appropriate for synthesis. However, most cells contain inorganic pyrophosphatase, which catalyzes the following highly exergonic reaction:

$$PP_i + H_2O \rightarrow P_i + P_i \qquad (23)$$

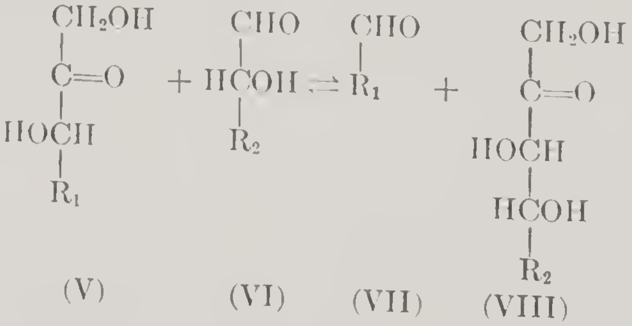
Thus, if Reactions 22 and 23 are coupled, synthesis of UDP-sugars can be obtained even starting with low concentrations of reactants.

b. Mutases. The position of the phosphate group joined to sugars may be changed by the action of enzymes. This action may be exemplified with the phosphoglucomutase reaction in the reversible transformation of glucose-6-phosphate (II) to glucose-1-phosphate (III). This reaction, which requires catalytic amounts of glucose-1,6-diphosphate (*50*), attains equilibrium when the reaction mixture contains 95% of the 6-phosphate. This equilibrium corresponds to a $\Delta F° = -1800$ cal. on going from the 1-phosphate to the 6-phosphate (*51*).

Reactions similar to that of phosphoglucomutase have been found to occur with the phosphates of acetylglucosamine (*52*), ribose (*53*), mannose (*54*), and galactose (*55*). In every case, activity is linked to the presence of catalytic amounts of the corresponding diphosphate.

c. Aldose-Ketose Transformation. Enzymes, usually called isomerases, catalyzing the reversible transformation of an aldose into a ketose ($R—CHOH—CHO \rightleftharpoons R—CO—CH_2OH$) are of widespread occurrence. The best known catalyzes the conversion of glucose-6-phosphate into fructose-6-phosphate, $\Delta F° = +400$ cal. Many others have been described as shown in Table II.

d. Transfer of C_2 Units: Transketolase. Transketolase catalyzes a transfer of the group $CH_2OH—CO—$ from a ketose to an aldose (Reaction 24). The result is that the ketose becomes an aldose with two carbon atoms less and the aldose becomes a ketose with two carbon atoms more.



$$\underset{(V)}{\begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ HOCH \\ | \\ R_1 \end{array}} + \underset{(VI)}{\begin{array}{c} CHO \\ | \\ HCOH \\ | \\ R_2 \end{array}} \rightleftharpoons \underset{(VII)}{\begin{array}{c} CHO \\ | \\ R_1 \end{array}} + \underset{(VIII)}{\begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ HOCH \\ | \\ HCOH \\ | \\ R_2 \end{array}} \qquad (24)$$

The substrates which have been found to be acted upon are shown in Table III. Transketolase seems to be an enzyme of universal occurrence. Its activity is linked with the presence of diphosphothiamine.

TABLE II

ISOMERASES

General reaction: R–CHOH–CHO $\rightleftharpoons$ R–CO–CH_2OH

Aldose	Ketose	Source	Reference
D-Mannose	D-Fructose	*Pseudomonas saccharophila*	*56*
D-Glucose-6-phosphate	D-Fructose-6-phosphate	Widespread	*57, 58*
D-Mannose-6-phosphate	D-Fructose-6-phosphate	Yeast, muscle	59
D-Arabinose	D-Ribulose	*Escherichia coli*	*60*
L-Arabinose	L-Ribulose	*Lactobacillus pentosus, Lactobacillus plantarum*	*61, 61a*
D-Xylose	D-Xylulose	Several bacteria	*62, 63, 64*
D-Lyxose	D-Xylulose	*Pseudomonas saccharophila*	*56*
D-Ribose-5-phosphate	D-Ribulose-5-phosphate	Widespread	*65*
D-Glyceraldehyde-3-phosphate	Dihydroxyacetone phosphate	Widespread	*66, 67*
D-Glycero-D-mannoheptose	D-Sedoheptulose	*Pseudomonas saccharophila*	*56*
D-Rhamnose	D-Rhamnulose	*Pseudomonas saccharophila*	*56*
L-Fucose	L-Fuculose	*Escherichia coli*	*68*
D-Glucuronate	D-Fructuronate	*Serratia marcescens*	*68a*
D-Galacturonate	D-Tagaturonate	*Escherichia coli*	*68b*

A process related to transketolase has been reported recently. It consists in a transfer of the group CH_2OH—CO— to inorganic phosphate, forming acetyl phosphate. The corresponding enzymes have been named phosphotransketolases. The phosphotransketolase from *Lactobacillus plantarum* (*69a*) has been found to act on D-xylulose-5-phosphate and that of *Acetobacter xylinum* (*69b*) on D-fructose-6-phosphate.

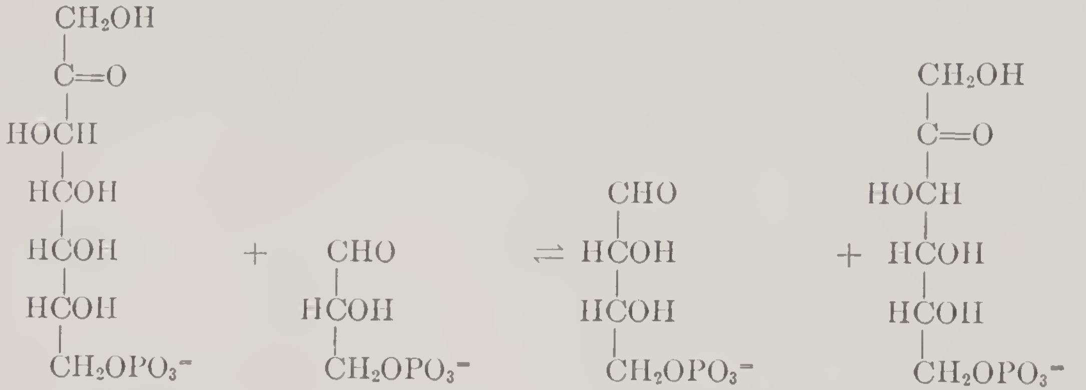
e. Transfer of C_3 Units: Transaldolase. A transfer of the dihydroxy-

TABLE III

TRANSKETOLASE (*12, 17, 69*)

Donor of CH_2OH–CO– ⟶	Product
D-Sedoheptulose-7-phosphate	D-Ribose-5-phosphate
D-Fructose-6-phosphate	D-Erythrose-4-phosphate
D-Xylulose	D-Glyceraldehyde
D-Xylulose-5-phosphate	D-Glyceraldehyde-3-phosphate
L-Erythrulose	Glycolaldehyde
Hydroxypyruvate	Carbon dioxide
Product ⟵	Acceptor of CH_2OH–CO–

acetone group $CH_2OH—CO—CHOH—$ is catalyzed by transaldolase. which is of widespread occurrence (Reaction 25).



$$\begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ HOCH \\ | \\ HCOH \\ | \\ HCOH \\ | \\ HCOH \\ | \\ CH_2OPO_3^{=} \end{array} + \begin{array}{c} CHO \\ | \\ HCOH \\ | \\ CH_2OPO_3^{=} \end{array} \rightleftharpoons \begin{array}{c} CHO \\ | \\ HCOH \\ | \\ HCOH \\ | \\ CH_2OPO_3^{=} \end{array} + \begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ HOCH \\ | \\ HCOH \\ | \\ HCOH \\ | \\ CH_2OPO_3^{=} \end{array} \qquad (25)$$

Sedoheptulose-7-phosphate (IX) | Glyceraldehyde-3-phosphate (X) | Erythrose-4-phosphate (XI) | Fructose-6-phosphate (XII)

Another reaction (*17*) which has been found to be catalyzed by enzymes of yeast and ascites tumor cells is

Octulose-8-phosphate + Glyceraldehyde-3-phosphate ⇄
Allose phosphate (?) + Xylulose-5-phosphate (26)

f. Aldolase. The enzyme aldolase has been studied thoroughly in connection with glycolysis. It catalyzes the reversible reaction:

Fructose-1,6-diphosphate →
Glyceraldehyde-3-phosphate + Dihydroxyacetone phosphate (27)

The equilibrium is displaced towards the left; thus, starting with 0.01 *M* fructose-1,6-disphosphate at pH 7.3, only 11% is transformed into triose phosphates. Aldolase preparations catalyze the condensation of many aldehydes with dihydroxyacetone phosphate as shown in Table IV.* Some of these reactions are undoubtedly artifacts obtained with unphysiological substrates, but others may occur in the cell.

Aldolase is present in plants, yeast, bacteria, and animal tissues. The enzyme from muscle has been crystallized (*88, 89*).

g. 1-Phosphofructoaldolase. An enzyme very similar to aldolase has been found in mammalian liver and muscle and in plants (*71, 73*). It has been partially separated from liver aldolase by ion exchange chromatography (*90*). The two enzymes differ in their specificity. Thus, the best substrate for aldolase is fructose-1,6-diphosphate, and that for 1-phosphofructoaldolase is fructose-1-phosphate. The reaction is as follows:

Fructose-1-phosphate ⇄ Dihydroxyacetone phosphate + D-Glyceraldehyde (28)

The equilibrium of this reaction is more displaced towards the left than that of aldolase, a result that has been ascribed to the fact that fructose-1-phosphate can exist in the pyranose form while only the furanose form is possible for fructose-1,6-diphosphate. It is known that pyranoses are thermodynamically more stable than furanoses.

---

* References *70–87a* are in Table IV.

Besides glyceraldehyde, other aldehydes can serve as substrates for the enzyme, and there is some doubt as to whether some of the reactions shown in Table IV are brought about by aldolase or by 1-phosphofructoaldolase (*77*).

TABLE IV
ALDOLASES

Aldehyde condensed	Product	Source	Reference
D-Glyceraldehyde-3-phosphate	D-Fructose-1,6-diphosphate	Widespread	*70*
D-Glyceraldehyde	D-Fructose-1-phosphate	Muscle, liver, plants	*70, 71, 72, 73*
Formaldehyde	D-Erythrulose-1-phosphate	Muscle, liver	*70, 74, 75*
Acetaldehyde	Methyltetrose-1-phosphate	Muscle	*70*
Propionaldehyde	?	Muscle	*70*
Butylaldehyde	?	Muscle	*70*
D,L-Lactaldehyde	6-Deoxy-D-fructose + 6-deoxy-L-sorbose	Muscle, plants	*70, 76*
Methylglyoxal	?	Muscle	*70*
Aldolphenylpropylaldehyde	?	Muscle	*70*
Glycolaldehyde	D-Xylulose-1-phosphate	Muscle, liver, plants	*70, 77, 78 79, 80*
Glycolaldehyde phosphate	D-Xylulose-1,5-diphosphate	Muscle, *Micrococcus pyogenes*	*78, 80*
D-Erythrose	D-Sedoheptulose-1-phosphate	Muscle, plants, liver	*77, 81, 82*
D-Erythrose-4-phosphate	D-Sedoheptulose-1,7-diphosphate	Muscle	*83*
D-Ribose-5-phosphate	D-Octulose-1,8-diphosphate	Muscle	*84*
D-Glyceraldehyde-3-phosphate[a]	D-Deoxyribose-5-phosphate	*Escherichia coli*, animal tissues	*85*
D-Glyceraldehyde-3-phosphate[b]	2-Keto-3-deoxy-6-phosphogluconate	Bacteria	*86, 87*
N-Acetyl-D-mannosamine[b]	*N*-Acetyl neuraminic acid	*Clostridium perfringes*	*87a*

[a] The other reactant is acetaldehyde.
[b] The other reactant is pyruvic acid.

The existence of the latter enzyme has been questioned recently by Peanasky and Lardy (*90a*), who crystallized liver aldolase and found that it catalyzes the splitting of fructose-1-phosphate.

h. Epimerization. Following the suggestion of Pigman (*1*), the term *epimers* is used here to mean any pair of stereoisomers that differ solely in the configuration of a single asymmetric carbon. Thus, mannose and glucose are 2-epimers and galactose and glucose are 4-epimers.

The epimerization reactions which are believed to be catalyzed by single enzymes are shown in Table V. Other reactions may take place by two step processes; for instance, in a 2-epimerization of mannose-6-phosphate to glucose-6-phosphate there is the intermediate formation of fructose-6-phosphate, two isomerases (Table II) being involved. Another example is offered by the 3,4-epimerization of D-xylulose to L-xylulose which takes place through reduction to xylitol (*100*).

TABLE V
EPIMERASES

Epimers		OH group inverted	Source	Reference
α-D-Glucose	β-D-Glucose	1	*Penicillium*, kidney	*91, 92*
D-Xylulose-5-phosphate	D-Ribulose-5-phosphate	3	Widespread	*93*
D-Xylulose-5-phosphate	L-Ribulose-5-phosphate	4	Widespread	*22, 94, 94a,b*
UDP-glucose	UDP-galactose	4	Yeast, bacteria, liver, blood, mammary gland	*36, 95, 96, 97 98, 98a*
UDP-acetylglucosamine	UDP-acetylgalactosamine	4	Liver, *Bacillus subtilis*	*98b,c*
UDP-acetylglucosamine	UDP-acetylmannosamine	2	Liver	*99, 99a*
UDP-D-xylose	UDP-L-arabinose	4	Mung bean	*47*
UDP-glucuronic acid	UDP-galacturonic acid	4	Plants, *Pneumococcus*	*99b,c*

The 3- and 4-epimerizations of D-xylulose-5-phosphate are believed to occur directly and no cofactor has been found necessary. In the case of the 4-epimerization of UDP-glucose DPN is required, so that presumably an oxidation is involved. This reaction will be dealt with in Section II, C, 2, *b*.

i. Oxido-reductions. (*1*) *Aldoses to onic acids.* The oxidation of aldoses in position 1 to yield onic acids has been extensively studied (*12, 69*), in particular the oxidation of glucose-6-phosphate, which is the first step of the so-called oxidative pathway:

$$\text{Glucose-6-phosphate} + \text{TPN} \rightarrow \text{6-Phosphogluconolactone} + \text{TPNH}_2 \qquad (29)$$

Many other reactions of the same type have been described: glucose to gluconic acid (TPN, liver) (*101*), L-arabinose to L-arabonolactone

(DPN, *Pseudomonas saccharophila*) (*102*), D-arabinose to D-*arabonolactone* (DPN, *P. saccharophila*) (*103*), D-galactose to D-galactonolactone (DPN, *P. saccharophila*) (*104*).

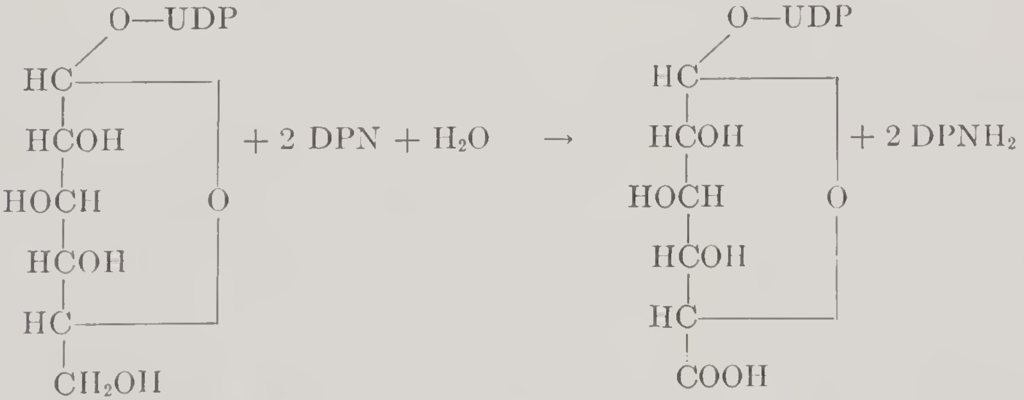
(*2*) *Polyols to ketoses.* Several enzymes are known which catalyze the reversible transformation of polyols to ketoses:

$$\mathrm{R{-}CHOH{-}CH_2OH \rightleftharpoons R{-}CO{-}CH_2OH}$$

The following have been described: D-sorbitol to D-fructose (DPN, kidney, liver and seminal vesicles) (*105, 106, 107*), several polyols to ketoses [DPN, *Acetobacter suboxydans* (*108*), *Pseudomonas* (*109*)], xylitol to L-xylulose (TPN, liver) (*110*), xylitol to D-xylulose (DPN, liver (*111*), ribitol to ribulose (*Aerobacter aerogenes*, DPN) (*111a*), D-mannitol-1-phosphate to D-fructose-6-phosphate (DPN, bacteria, yeast) (*112, 113*).

(*3*) *Aldoses to polyols.* Reduction of the aldehyde group of aldoses yields polyols. The enzymes known are: D-glucuronate to L-gulonate (a step in ascorbic acid synthesis) (*114*), D-glucose to D-sorbitol (TPN, seminal vesicles, placenta) (*107*), and D-xylose to D-xylitol (TPN, *Penicillium chrysogenum*) (*114a*).

(*4*) *Formation of uronic acids.* The oxidation of the primary alcohol group at carbon 6 of aldoses to a carboxyl group leads to the formation of an uronic acid. Such an oxidation is catalyzed by an enzyme which acts on UDP-glucose (Reaction 30).



(30)

UDP-glucose (XIII)

UDP-glucuronic acid (XIV)

Only one enzyme seems to be involved, although the process should consist of two steps as follows:

$$\mathrm{R{-}CH_2OH \rightarrow RCHO \rightarrow RCOOH}$$

It has not been possible to detect the aldehyde intermediate.

The enzyme from liver has been purified and studied thoroughly (*115, 116*). UDPG-dehydrogenase has also been detected in pea seedlings (*117*), and *Pneumococcus* (*117a*).

(5) *Other reactions.* With extracts from *Pseudomonas saccharophila* an enzymatic internal oxido-reduction of 6-phosphogluconic acid yields 2-keto-3-desoxy-6-phosphogluconic acid, which is further transformed to pyruvic acid and glyceraldehyde-3-phosphate (*118*). Similar reactions have been detected in the same organism with galactonic acid (*104*) and D-arabonic acid (*103*). 6-Phosphogluconic acid can be oxidized to D-ribulose-5-phosphate and CO_2 (*12, 69*).

2. *Some Cases of Interconversion*

The manifold reactions described in the previous section may be combined in different manners, so that the same product may be formed by several pathways. Which pathways are the main routes in each organism is a question which must remain unanswered for some time. Some examples of combinations of the general reactions will be considered in what follows.

a. Glucose to Fructose. Glucose-6-phosphate is converted into fructose-6-phosphate by the enzyme phosphoglucoisomerase, which has been detected in many different organisms. If a phosphatase is present, fructose-6-phosphate gives rise to free fructose. It was believed that the free fructose found in seminal vesicles and in the fetal blood of ungulates arises by this mechanism. However, further work showed that the interconversion occurs according to the following sequence:

$$\text{Glucose} \xrightarrow{\text{TPNH}_2 \quad \text{TPN}} \text{Sorbitol} \xrightarrow{\text{DPN} \quad \text{DPNH}_2} \text{Fructose} \qquad (31)$$

Two enzymes are involved: ketose reductase and aldose reductase. According to the distribution of the enzymes, it seems that both steps take place in the seminal vesicles, while only the first would occur in placenta. The sorbitol thus produced would be transported via blood to the liver where it would be oxidized to fructose (*107, 119*).

b. Galactose. Galactose is phosphorylated in position 1 by ATP, in the presence of a kinase contained in mammalian liver and microorganisms (Table I). Glucose and galactose phosphates are interconverted according to Reactions 32–34.

$$\text{Galactose-1-phosphate} + \text{UDP-glucose} \rightleftharpoons \text{Glucose-1-phosphate} + \text{UDP-galactose} \qquad (32)$$

$$\text{UDP-galactose} \rightleftharpoons \text{UDP-glucose} \qquad (33)$$

$$\textit{Sum:} \quad \text{Galactose-1-phosphate} \rightleftharpoons \text{Glucose-1-phosphate} \qquad (34)$$

Reaction 32 is catalyzed by an enzyme which has been named galactose-1-phosphate-uridyl transferase, and Reaction 33 is catalyzed by UDP-glucose epimerase (*35, 36, 120, 121*). Both enzymes have been detected in yeast (*36*), bacteria (*95, 96*), liver (*97*), and blood (*98*), but only the epimerase in mammary gland (*97*). In some human subjects, galactose-1-phosphate-uridyl transferase is lacking (*98*), and this explains their inability to metabolize galactose.

The 4-epimerization of UDPG (Reaction 33) requires the presence of DPN (*120*). It is therefore likely that the inversion of configuration takes place through an oxidation by DPN and a subsequent reduction to the epimeric sugar derivative. This mechanism is consistent with the finding that the elements of labeled water do not exchange with the glucose or galactose bound to UDP (*122, 123, 124*).

The equilibrium of the over-all reaction (34) is attained when the respective concentrations of the galactose and glucose phosphates are in the proportion 1:3, giving a $\Delta F^\circ = -700$ cal. (*125, 126*).

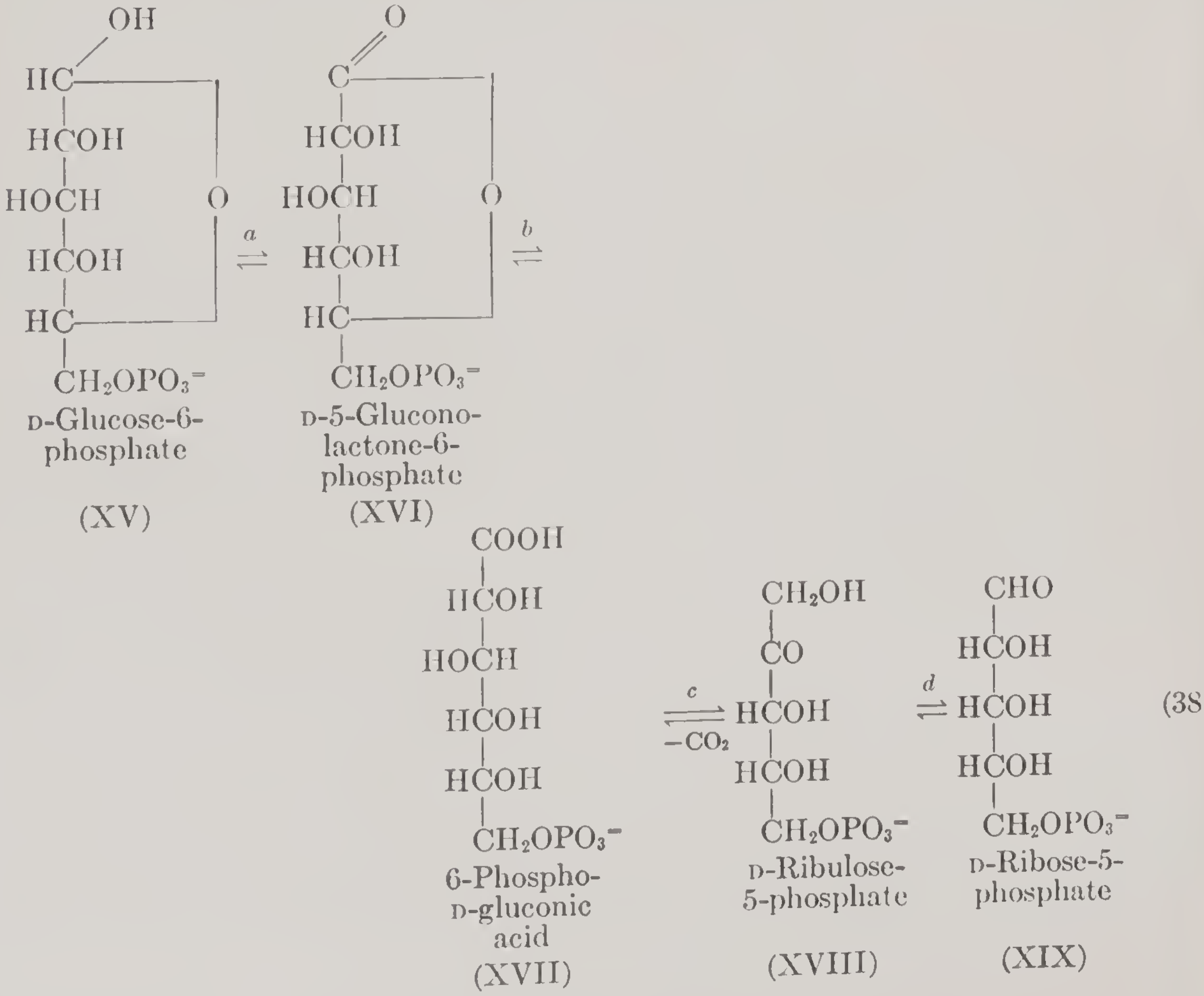
While Reactions 32 and 33 describe the utilization of galactose as a carbon source, the formation of galactose derivatives probably occurs according to the following sequence:

$$\text{UTP} + \text{Glucose-1-phosphate} \rightleftharpoons \text{UDP-glucose} + \text{PP}_i \quad (35)$$

$$\text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} \quad (36)$$

$$\text{UDPG-galactose} + \text{Acceptor} \rightleftharpoons \text{UDP} + \text{Galactose-acceptor} \quad (37)$$

This has been shown to be the case for the synthesis of lactose, as it will be seen later. It will be noticed that this pathway does not involve the formation of free galactose or galactose-1-phosphate.



D-Glucose-6-phosphate (XV)

D-5-Glucono-lactone-6-phosphate (XVI)

6-Phospho-D-gluconic acid (XVII)

D-Ribulose-5-phosphate (XVIII)

D-Ribose-5-phosphate (XIX)

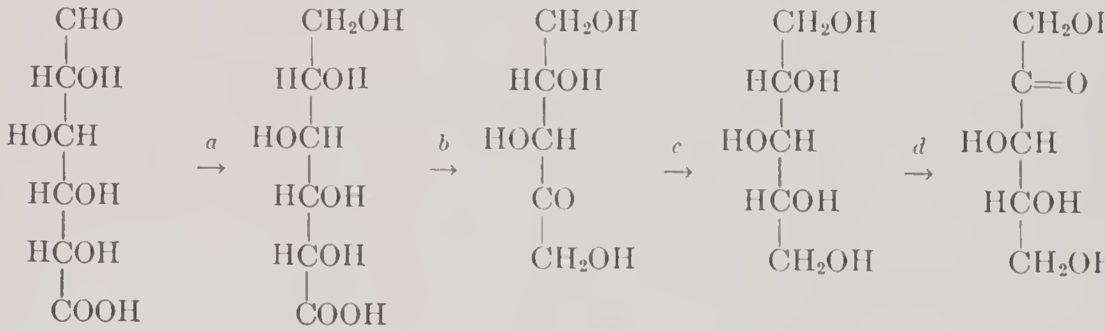
(38)

c. The Formation of Pentoses. There are several pathways which can lead to the formation of pentoses. One of the mechanisms is the oxidation of glucose-6-phosphate as shown in Reaction 38.

The corresponding enzymes have been found in animals, plants and microorganisms (*12, 69*). Steps *a* and *c* are dehydrogenations with TPN as oxidant, step *b* is an opening of the lactone ring, and step *d* is an isomerization. The whole process is reversible.

Pentose phosphates can also be formed by the action of transketolase. Two carbon atoms are added to D-glyceraldehyde phosphate, giving D-xylulose-5-phosphate. The donor of the two carbon unit may be sedoheptulose-7-phosphate or fructose-6-phosphate, leaving respectively D-ribose-5-phosphate or erythrose-4-phosphate (see Table III).

Other enzymes which bring about the formation of pentoses have been detected in animal tissues. Thus, starting from glucuronic acid which is known to be formed from glucose (through the UDP-derivative), D-xylulose can be formed as shown in Reaction 39.



(39)

D-Glucuronic acid (XX)
L-Gulonic acid (XXI)
L-Xylulose (XXII)
Xylitol (XXIII)
D-Xylulose (XXIV)

It should be noted that the L-sugars are written upside down, that is, with position 1 at the bottom. Step *a*, which is a reduction of the aldehyde group of D-glucuronic acid, is also necessary for ascorbic acid synthesis (*114*). All these reactions have been detected using liver enzymes. Step *b* (*127*) is similar to the oxidation of D-phosphogluconic acid to D-ribulose-5-phosphate. Steps *c* and *d* are oxido-reductions with $TPNH_2$ and DPN respectively (*100*). L-Xylulose is excreted in urine and is known to arise from glucuronic acid (*128*). Presumably, the pathway is that shown in steps *a* and *b* in Reaction 39. In plants, experiments with labeled sugars have shown that D-xylose (isolated from the xylan) is formed from D-glucose by loss of carbon atom 6 (*129*). This change can be written on paper as a decarboxylation of UDP-glucuronic acid to UDP-D-xylose followed by xylan synthesis from the latter. Recent work supports this assumption. It was found that extracts of asparagus catalyze the decarboxylation of UDP-glucuronic acid to UDP-pentose (xylose and arabinose) (*99b*). Plants (*117*) and bacteria

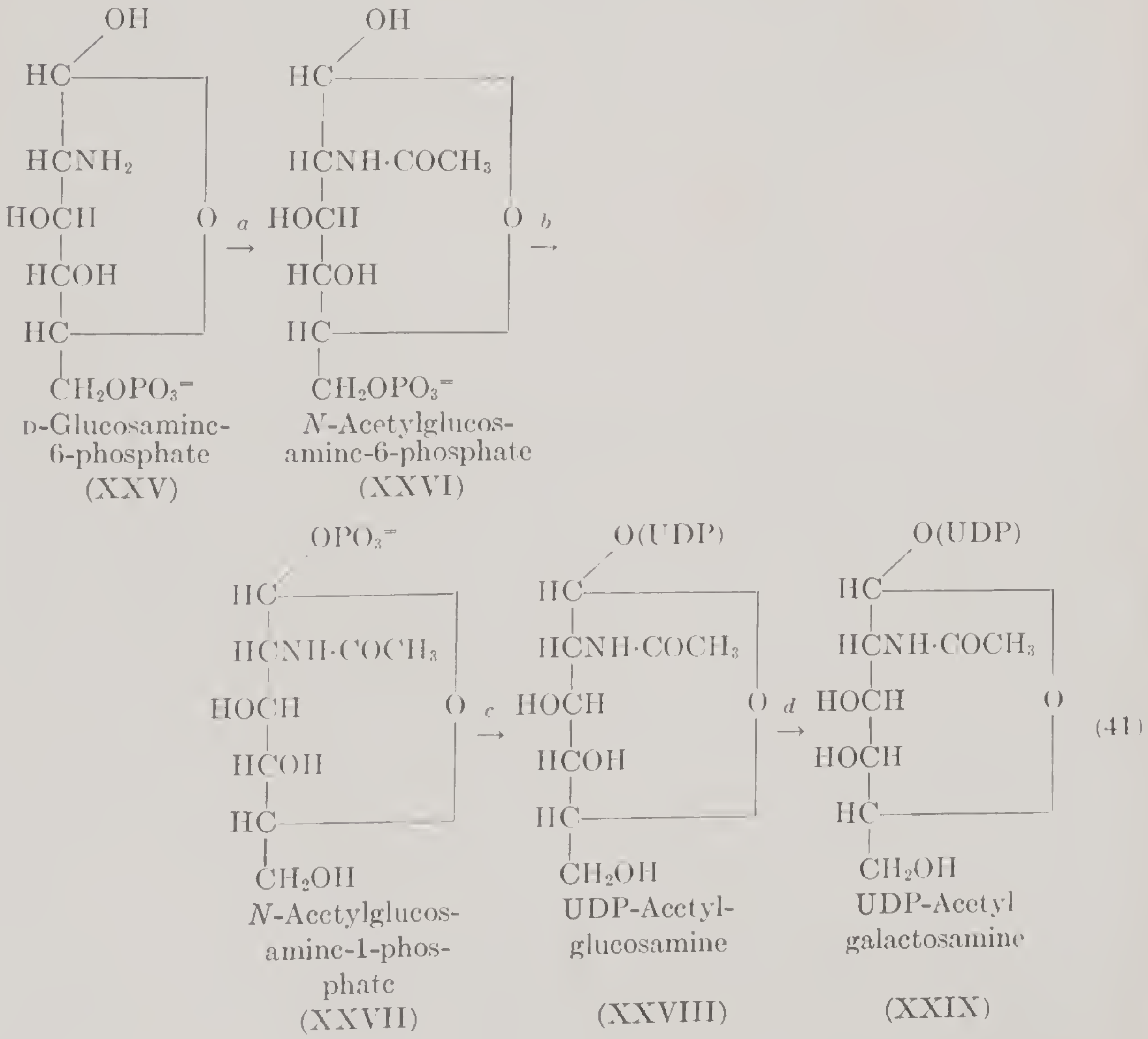
(*117a*) can transform UDPG into UDP-glucuronic acid and the latter into UDP-galacturonic acid (*99b,c*). Decarboxylation of UDP-galacturonic would yield UDP-L-arabinose which can also be formed by 4-epimerization of UDP-D-xylose (*47*).

Two other mechanisms of synthesis of pentoses can be mentioned. One is the condensation of dioxyacetone phosphate with glycolaldehyde, catalyzed by aldolase, which yields D-xylulose-1-phosphate (see Table IV). The other synthesis consists in a condensation of formaldehyde with tetrulose phosphate giving a pentose phosphate (*130*). The enzyme has been found in animal tissues.

d. Aminosugars. Enzymes have been obtained from molds (*Neurospora*) (*29*) and animal tissues (liver) (*131*) which catalyze the formation of glucosamine-6-phosphate (XXV) as follows:

Hexose-6-phosphate + Glutamine ⇌ Glucosamine-6-phosphate + Glutamic acid (40)

Several other enzymes can act on glucosamine-6-phosphate according to Reaction 41.


D-Glucosamine-6-phosphate
(XXV)
N-Acetylglucosamine-6-phosphate
(XXVI)
N-Acetylglucosamine-1-phosphate
(XXVII)
UDP-Acetylglucosamine
(XXVIII)
UDP-Acetyl galactosamine
(XXIX)
a
b
c
d

(41)

These reactions consist of (*a*) acetylation (*29, 132*), (*b*) migration of the phosphate group (*52*), (*c*) reaction with UTP to give UDP-acetylglucosamine (*46*), and (*d*) 4-epimerization (*98b,c*). The 2 UDP derivatives formed are believed to be involved in mucopolysaccharide synthesis.

Another enzyme, which has been obtained from several sources (*133–135a*), catalyzes the following reaction:

$$\text{Glucosamine-6-phosphate} + H_2O \rightleftharpoons \text{Fructose-6-phosphate} + NH_3 \qquad (42)$$

Since the reaction is reversible, it might be used for the synthesis of glucosamine-6-phosphate *in vivo*.

Liver has been found to contain an enzyme system which leads to the formation of free acetylmannosamine from UDP-acetylglucosamine (*99, 99a*).

III. The Formation of Glycosidic Bonds (*136*)

A. INTRODUCTION

The different monosaccharide units formed by the various interconversion reactions may be condensed to di-, oligo-, or polysaccharides. The linkage between the units takes place between a hemiacetal hydroxyl group (position 1 in aldoses or position 2 in ketoses) and another hydroxyl group. The latter may be also a hemiacetal group, as in nonreducing disaccharides (sucrose and trehalose) or a primary or secondary alcohol group.

Polysaccharides can be constituted by units of the same or of different sugars, and on this basis they are classified as homo- or heteropolysaccharides. These groups can be further subdivided according to the type of linkage. Thus, the glucans can have $\alpha(1 \rightarrow 4)$ linkages as in amylose, or $\beta(1 \rightarrow 4)$ as in cellulose.

Heteropolysaccharides can have two kind of sugars, as some mucopolysaccharides which contain alternating units of hexosamine and uronic acid, or may contain several different monosaccharides, as occurs with blood group substances and with plant gums.

The general mechanism by which monosaccharide units are transformed into oligo- and polysaccharides consists in a transfer reaction in which a glycosyl derivative acts as monosaccharide donor to an acceptor as follows:

$$\text{Glycosyl—R} + \text{Acceptor} \rightarrow \text{Glycosyl-acceptor} + \text{R} \qquad (43)$$

The group R may be a phosphate group, a nucleoside pyrophosphate group such as uridine diphosphate, or a saccharide as in the different transglycosylations. The acceptor may be a monosaccharide, when disaccharides are synthesized, or a polysaccharide chain which becomes elongated in the process.

It is by one of these mechanisms or by a combination of them that the numerous saccharides found in nature are synthesized (Fig. 2).

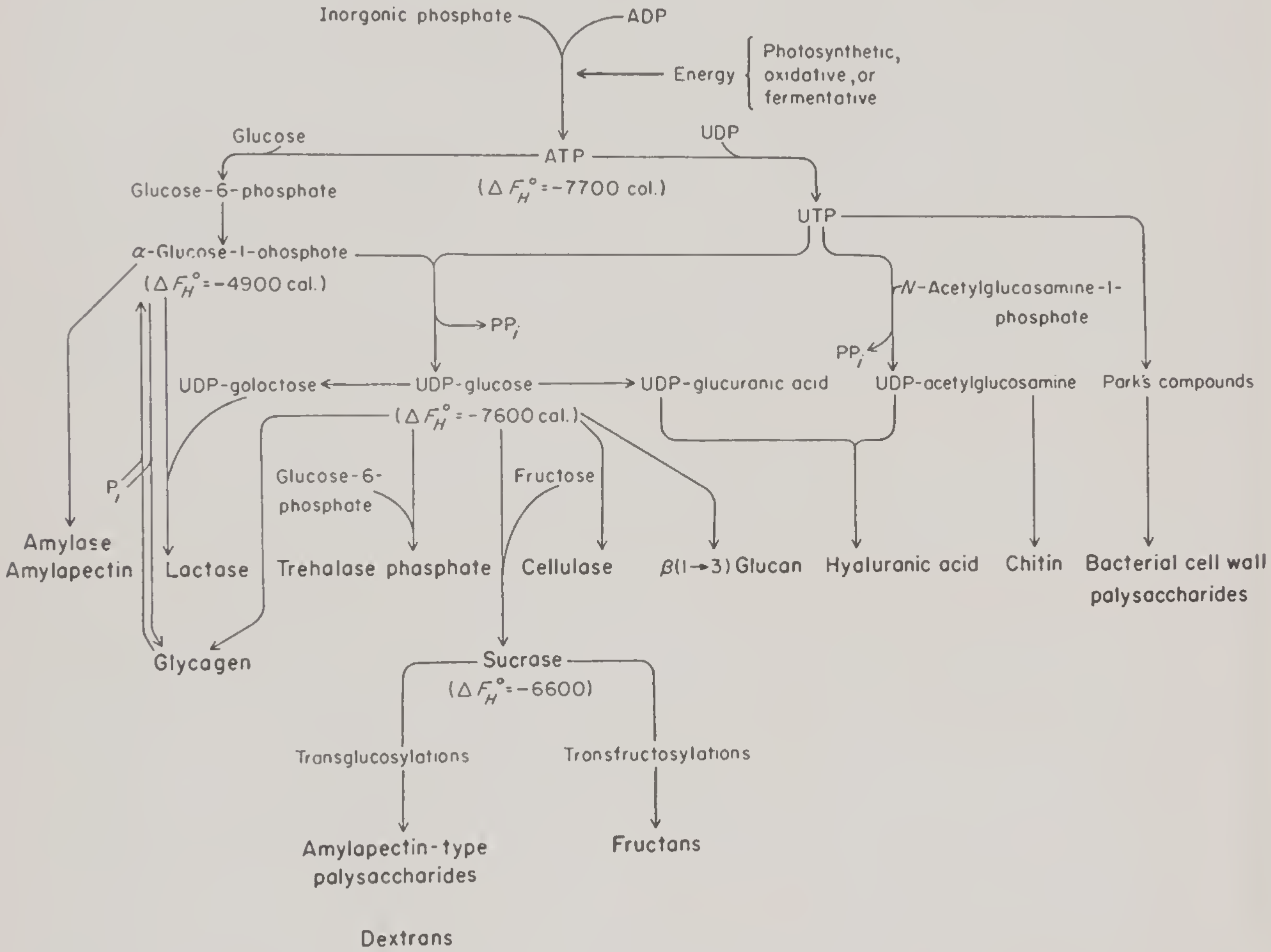


FIG. 2. Synthesis of polysaccharides.

Studies in this field are only at the beginning and it is likely that other mechanisms, still unknown, are also involved.

The mechanism of formation of the 1-sugar phosphate required for some of these synthesis follows some of the pathways mentioned in the previous section. For instance:

$$\text{Glucose} \xrightarrow{\text{ATP}} \text{Glucose-6-phosphate} \rightarrow \text{Glucose-1-phosphate} \tag{44}$$

$$\text{Galactose} \xrightarrow{\text{ATP}} \text{Galactose-1-phosphate} \tag{45}$$

$$\text{Acetylglucosamine-6-phosphate} \longrightarrow \text{Acetylglucosamine-1-phosphate} \tag{46}$$

The 1-sugar phosphates may act directly as glucose donors for saccharide synthesis or may be converted to UDP derivatives by reacting with UTP as previously described in Section II, C, 1, *a*.

The glycosidic bonds formed from sugar phosphates or from the UDP derivatives may be redistributed by various enzymes known as transglycosylases, which catalyze the transfer of glycosyl groups from a donor molecule to an acceptor. When the acceptor is water, the result is hydrolysis. Many, or perhaps all, the enzymes which were previously considered to be hydrolytic produce a variable amount of transglycosylation to different acceptors. This is somewhat surprising, because water is always present at a much greater concentration (55 *M* for pure water) than any other substance.

Some of the glycosyl group transferring enzymes do not produce appreciable hydrolysis, that is, they do not use water as an acceptor; others catalyze both reactions to variable degrees. Indeed, there may be a whole spectrum of actions ranging from pure hydrolases to pure transferases.

In the reactions catalyzed by enzymes which are not very specific for the acceptor, it is usually the primary alcohol group which becomes substituted. This preferential reactivity of the primary alcohol is also apparent in many organic reactions (*137*).

In all the cases which have been studied, the bond broken during the transfer reactions is that between the carbon atom and oxygen. That is, a glycosyl group is transferred and hence the name *transglycosylase* and not *transglycosidase* which would imply the breaking of the bond beyond the oxygen (*138, 139, 140*).

B. Free Energy Changes

The values available in the literature for the free energy of hydrolysis of some compounds are as follows: sucrose, —6600 cal. (*141*); glucose-1-phosphate (at pH 8.5 and 38°C), —4900 cal. (*142*); lactose, about —3000 cal. (*143*), maltose, about —3000 cal. (*144*); glycogen [$\alpha(1 \rightarrow 4)$-linkages], approximately —4300 cal. (*145*); levan [fructofuranosyl $\beta(2 \rightarrow 6)$-linkages], —4600 cal. (*146*); dextran (glucopyranosyl $\alpha(1 \rightarrow 6)$-linkages), —2000 cal. or less (*144*).

A value for UDP-glucose can be calculated by adding to the $\Delta F_H{}^\circ$ of sucrose, the ΔF° of the sucrose synthesis reaction [about —1000 cal. (Sect. III,C,1)]. This would give a $\Delta F_H{}^\circ = -7600$ cal. for the hydrolysis of the glucose group at pH 7.4 and 37°. This value should change with pH, since a secondary phosphoric acid group becomes unmasked in the hydrolysis. The corresponding ΔF° would be about —1800 cal. in going from pH 6 to 8 (*142*).

It should be noted that several of the ΔF's given above have not been corrected for pH effects depending on the different pK's of reactants and products. These corrections would not change substantially the values given, and it is doubtful whether their inclusion is justified, since some of the measurements are probably not very accurate. With all the uncertainties which these values contain it may be concluded that the glucose transfer potential decreases in the order UDP-glucose, sucrose, glucose-1-phosphate.

C. Synthesis of Disaccharides

1. *Sucrose* (*147, 148*)

Sucrose synthesis may be carried out in plant tissues by two similar mechanisms (*149–152*). One enzyme acts on free fructose as follows:

$$\text{UDPG} + \text{Fructose} \rightleftharpoons \text{UDP} + \text{Sucrose} \qquad (47)$$

The reaction is reversible and displaced towards sucrose synthesis, but accurate values for the equilibrium constant have not been obtained. At pH 7.4 and 37° the values obtained correspond to a ΔF° of about -1000 cal. (*150*). It may be mentioned here that another enzyme which has been obtained from bacteria (*Pseudomonas*), and named sucrose phosphorylase, catalyzes a similar reaction but starting with glucose-1-phosphate (*147, 148*):

$$\text{Glucose-1-phosphate} + \text{Fructose} \rightleftharpoons \text{Sucrose} + \text{P}_i \qquad (48)$$

In this case the reaction is displaced towards the phosphorolysis of sucrose. The values correspond to $\Delta F^\circ = +1770$ cal. at pH 6.6 and 30° (*141*).

The other plant enzyme (*152*) which can lead to sucrose synthesis catalyzes the reaction:

$$\text{UDGP} + \text{Fructose-6-phosphate} \rightleftharpoons \text{UDP} + \text{Sucrose phosphate} \qquad (49)$$

The equilibrium has not been determined. In this case, the reaction can be pulled further towards synthesis by adding the reaction:

$$\text{Sucrose phosphate} \rightarrow \text{Sucrose} + \text{P}_i \qquad (50)$$

Therefore, with such a reaction system it should be possible to build up high sucrose concentrations as found in some plants such as sugar cane and beet.

Experiments with labeled compounds are consistent with such a mechanism of sucrose synthesis (*149, 151, 153*).

2. *Trehalose*

Fungi, yeast, and insects contain the disaccharide trehalose (1-O-α-D-glucopyranosyl-α-D-glucopyranoside). It is also found in some plants where it is perhaps produced by association with insects.

The synthesis of trehalose has been found to be catalyzed by a yeast enzyme (*154, 155*), according to the reaction:

$$\text{UDPG} + \text{Glucose-6-phosphate} \rightarrow \text{Trehalose phosphate} + \text{UDP} \qquad (51)$$

A specific phosphatase hydrolyzes the product as follows:

$$\text{Trehalose phosphate} \rightarrow \text{Trehalose} + \text{P}_i \tag{52}$$

3. *Lactose*

Lactose (4-O-β-D-galactopyranosyl-D-glucopyranose) is formed in the mammary gland and has also been detected in some plants: *Forsythia* (*156*) and *Sapotacea* (*157*). Studies on its mechanism of synthesis with enzymes extracted from the mammary gland have shown that it is formed from UDP-galactose and glucose-1-phosphate (*158*). Therefore, a series of known reactions (53–61) leading from glucose to lactose may be written.

$$2\ \text{ATP} + 2\ \text{Glucose} \rightarrow 2\ \text{Glucose-6-phosphate} + 2\ \text{ADP} \tag{53}$$

$$2\ \text{Glucose-6-phosphate} \rightarrow 2\ \text{Glucose-1-phosphate} \tag{54}$$

$$\text{UTP} + \text{Glucose-1-phosphate} \rightarrow \text{UDP-glucose} + \text{PP}_i \tag{55}$$

$$\text{UDP-glucose} \rightarrow \text{UDP-galactose} \tag{56}$$

$$\text{UDP-galactose} + \text{Glucose-1-phosphate} \rightarrow \text{Lactose-1-phosphate} + \text{UDP} \tag{57}$$

$$\text{Lactose 1-phosphate} + \text{H}_2\text{O} \rightarrow \text{Lactose} + \text{P}_i \tag{58}$$

$$\text{ATP} + \text{UDP} \rightarrow \text{UTP} + \text{ADP} \tag{59}$$

$$\text{PP}_i + \text{H}_2\text{O} \rightarrow 2\ \text{P}_i \tag{60}$$

$$\textit{Sum:}\quad 3\ \text{ATP} + 2\ \text{Glucose} + 2\ \text{H}_2\text{O} \rightarrow \text{Lactose} + 3\ \text{ADP} + 3\ \text{P}_i \tag{61}$$

Three molecules of ATP become hydrolyzed in the synthesis of one lactose molecule. The ΔF° of hydrolysis of 3 moles of ATP is −23,000 cal., and that of lactose about −3000 cal. Evidently, the process does not appear to be very efficient from the thermodynamic point of view.

4. *Maltose and Cellobiose*

Maltose and cellobiose are formed by the action of hydrolytic enzymes on starch or cellulose respectively. The two disaccharides can be degraded by phosphorolysis according to the following equations:

$$\text{Maltose} + \text{P}_i \rightleftharpoons \beta\text{-Glucose-1-phosphate} + \text{Glucose} \tag{62}$$

$$\text{Cellobiose} + \text{P}_i \rightleftharpoons \alpha\text{-Glucose-1-phosphate} + \text{Glucose} \tag{63}$$

These two reactions are reversible, as is the one mentioned for sucrose (Reaction 48), so that they may lead to the synthesis of disaccharides from glucose-1-phosphate and glucose. However, it is believed that *in vivo* they are used not for synthesis but for dissimilation. An interesting feature of these processes is that maltose, which has an $\alpha(1 \rightarrow 4)$-linkage gives the β-phosphate-phosphate ester, while the inverse holds true for cellulose, which gives the α-ester from $\beta(1 \rightarrow 4)$-linkages. The enzymes have been obtained from bacteria: maltose phosphorylase in *Neisseria meningitidis* (*159*) and cellobiose phosphorylase in *Clostridium thermocellum* (*160*).

D. Synthesis of Oligosaccharides (*161, 162*)

Numerous oligosaccharides have been isolated from plants, several of them being substitution products of sucrose [gentianose, raffinose, stachyose and melezitose (Fig. 3)]. Others such as 1-kestose, 6-kestose, and neokestose have been obtained by enzyme action *in vitro*. Some of these oligosaccharides are formed by the action of invertases. These enzymes catalyze the hydrolysis of sucrose to glucose and fructose and also a certain amount of transglycosylation (*163, 164, 165*). With yeast invertase, the amount of tri- or higher saccharides never exceeds 10% of the total amount of sugar. These oligosaccharides are also hydrolyzed by the enzyme, so that they occur transiently in the reaction mixture. The transferring action is considered to be brought about by the hydrolyzing enzyme, because both activities run parallel at different stages of purification.

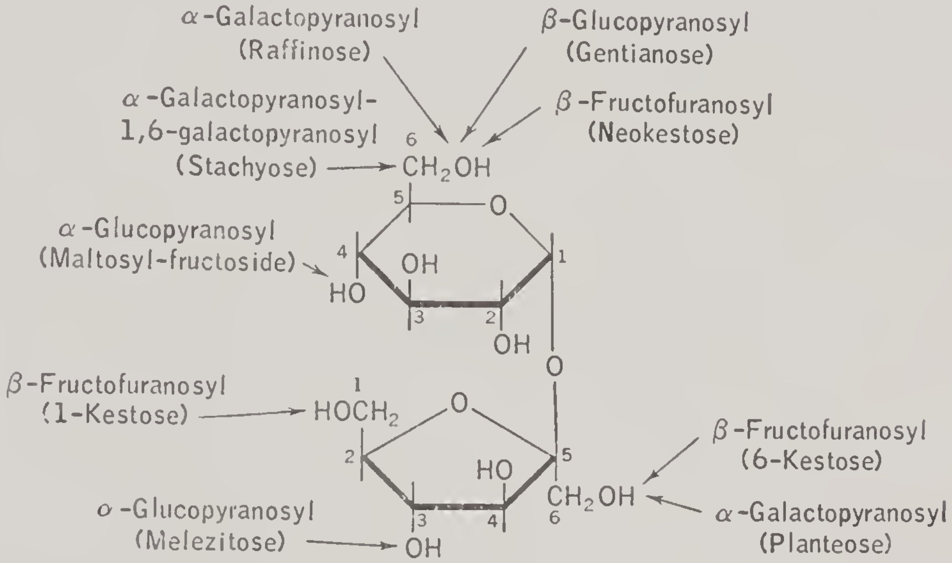


Fig. 3. Structure of some oligosaccharides derived from sucrose. Introduction of the different substituents at the positions indicated by the arrows gives the oligosaccharides mentioned in parenthesis. For instance, introduction of a β-glucopyranosyl group at position 6 of the glucose moiety of sucrose gives gentianose.

Yeast invertase catalyzes the transfer of β-fructofuranosyl residues from several donors such as sucrose, raffinose or methyl-β-D-fructofuranoside. The list of acceptors is longer since it includes most substances containing a primary alcohol group: e.g., hexoses (glucose, galactose, mannose), disaccharides (sucrose), sugar alcohols (sorbitol, mannitol), and other alcohols (methyl, ethyl, butyl, etc.).

Another invertase which has been studied carefully is that of molds (*Aspergillus* and *Penicillium*) (*166*). It differs in certain properties from yeast invertase. Thus, although mold invertase also catalyzes hydrolysis and the transfer of β-fructofuranose residues, it can produce a higher concentration of oligosaccharides (up to 50%). Moreover, the two invertases are affected in different ways by monosaccharides. The hydrolysis of sucrose by mold invertase is inhibited by glucose, and so is the formation of higher saccharides. On the other hand, hydrolysis by yeast invertase is more inhibited by fructose than by glucose. Other differences were detected using labeled hexoses. The mold enzyme catalyzed the incorporation of the label of free glucose into sucrose, while this did not occur with the yeast enzyme. The rationalization of these findings has not been carried out as yet.

Experiments with invertases of higher plants have shown that they also catalyze fructose transfers (*167*).

Although most invertases transfer fructose residues, some cases are known in which the glucose residue is transferred. This is the case with honey invertases (*168*), which can catalyze the following reactions:

$$\text{Sucrose} + \text{Glucose} \rightarrow \text{Fructose} + \text{Maltose} \qquad (64)$$

$$\text{Sucrose} + \text{Sucrose} \rightarrow \text{Fructose} + \text{Maltosyl fructoside} \qquad (65)$$

Several other oligosaccharides have been detected as products of the action of honey invertase on sucrose and on maltose.

It is not clear whether the honey enzymes are produced by the bee or are derived from flowers, because they may be detected both in plants and in insects. Thus, a transglycosylase has been found in the nectar of flowers of *Robinia pseudo-acacia* (*169*). Insects, on the other hand, are involved in the formation of some oligosaccharides associated with plants. The trisaccharide melezitose [O-α-D-glucopyranosyl(1 → 3)-O-β-D-fructofuranosyl(2 → 1)α-D-glucopyranoside] occurs in mannas and honey dews. Its formation is associated with the activity of sucking insects (Hemiptera) such as aphids (*170*). Extracts of some of these insects (*Eucallipterus tiliae* L.) have been found to form melezitose from sucrose. The reaction consists in the transfer of a glucose residue from sucrose to position 3 of the fructose moiety of another sucrose.

Other aphids (*Aphis spiraecola*) produce maltosyl fructoside [O-α-D-glucopyranosyl- (1 → 4) -O-α-D-glucopyranosyl- (1 → 2) -β-D-fructofuranoside] from sucrose. In this reaction, which is also catalyzed by honey enzymes, the glucose residue is transferred to position 4 of the glucose moiety of sucrose. The different products formed by insects have been proposed as a criterion for classification. The scale *Coccus hesperidum* L. excretes maltosyl fructoside together with the enzyme that forms it from sucrose, while *Icerya purchasi* excretes melezitose and the corresponding enzyme.

Enzymes obtained from molds catalyze the transfer of glucose residues from maltose (4-O-α-D-glucopyranosyl-D-glucose). For instance, extracts of *Aspergillus niger* catalyze the reaction:

$$2 \text{ Maltose} \rightarrow \text{Panose} + \text{Glucose} \qquad (66)$$

The glucose residue from a maltose molecule is transferred to position 6 of the glucosyl residue of another maltose giving panose (*171, 172*) (O-α-D-glucopyranosyl-(1 → 6)-O-α-D-glucopyranosyl(1 → 4)-D-glucopyranose).

The extracts also catalyze the transfer to position 3 of free glucose, forming the disaccharide nigerose (3-O-α-D-glucopyranosyl-D-glucopyranose) (*173*). In this connection it may be mentioned that *A. niger* contains a polysaccharide, nigeran, which is formed by glucose residues joined alternatively by 1 → 4 and 1 → 3 linkages.

As to galactose-containing oligosaccharides, some have been obtained by using lactose as donor (*174, 175, 176*), and raffinose has been prepared by transgalactosylation from α-phenylgalactoside to sucrose using an enzyme obtained from coffee seeds (*177*). Future work will have to decide whether the above-mentioned mechanisms of oligosaccharide synthesis occur in nature or whether other reactions are involved.

E. SYNTHESIS OF POLYSACCHARIDES (*138, 178, 179*)

1. *Synthesis of Glucans*

a. Glycogen and Starch. (*180–185*) One of the most carefully studied processes is the synthesis of glycogen or starch by the joint action of polysaccharide phosphorylase and a branching enzyme. More recent studies have shown that UDPG can be used as glucose donor in the synthesis of glycogen.

(*1*) *Phosphorylases.* Glycogen consists in a ramified chain of α-glucopyranose units joined by 1 → 4 linkages, with branches joined by 1 → 6 linkages. It has a tree-like structure, the average length of the branches being 12 units. While animal tissues contain glycogen, plants contain starch which is an α-glucopyranose polymer made up of amylopectin and amylose. The latter is formed by long linear chains of glucose units joined 1 → 4, while amylopectin resembles glycogen in being ramified, but the average length of the branches is longer, ranging from 18 to 27 units (*185*).

The discovery of the mechanism by which glycogen and starch are formed *in vivo* is of the greatest historical importance, not only on account of the fundamental nature of these materials in the economy of cells, but also because this was among the first examples of an "anabolic" process taking place *in vitro*. It showed how the energy which is liberated in oxidative reactions and trapped in ATP could be used to create new linkages through a chain of transfer reactions, first of phosphate and then of glucose. The breakdown of glycogen was studied before its synthesis, a not too uncommon sequence in biochemistry.

The synthesis of glycogen or starch from glucose-1-phosphate with soluble enzymes, obtained from cells of yeast (*186*), animals (*187*), or plants (*58*), was reported between 1939 and 1940.

TABLE VI

OCCURRENCE OF PHOSPHORYLASE[a]

Source	Reference
Vertebrate tissues	
Brain (calf, rabbit)	*186, 187, 188*
Cartilage (rabbit, rat)	*189, 190*
Fat (human, rat, rabbit)	*191*
Heart (calf, rabbit, rat)	*186, 187*
Leukocytes (dog, horse)	*192*
Liver (calf, rabbit)	*186, 187, 193, 194*
Muscle (calf, carp, cat, frog, rabbit, rat)	*187, 188, 195, 196, 197*
Retina (bovine)	*198*
Subcutaneous tissue (rat)	*199*
Wool roots (sheep)	*200*
Invertebrates	
Nematodes	*201*
Plant tissues	
Germinating seed (soybean)	*202*
Leaves (geranium, beet)	*58, 202, 203*
Plastids (various plants)	*204*
Pollen (waxy maize)	*205*
Roots (sugar beet)	*58, 203*
Seeds (barley, pea, maize, Lima beans, broad beans, jack beans, green gram)	*58, 206, 207, 208, 209, 210, 211*
Rhizomes (potato)	*207*
Microorganisms	
Bacteria (*Corynebacterium diphtheriae*, streptococci, *Neisseria perflava, Clostridium oedematiens*)	*212, 213, 214, 215, 216*
Protozoa (*Polytomella cocca*)	*217*
Yeasts (*Saccharomyces cerevisiae, Torulopsis rotundata*)	*218, 219, 220*

[a] Adapted from Hehre, 1951 (*138*).

Table VI shows the widespread occurrence of the enzyme. Muscle (*221*) and potato (*222*) phosphorylases have been isolated in the crystalline form.

Although the reaction catalyzed by polysaccharide phosphorylases of different origin is the same, there are some variations in specificity regarding the acceptor molecule. A primer is always required. For the muscle enzyme it has to be a large branched molecule (*223*), while for the potato enzyme linear tri- or tetrasaccharides are sufficient (*224*). Phosphorylase catalyzes a stepwise transfer of glucosyl units, from α-glucose-1-phosphate to the nonreducing ends of the primer (see Fig. 4), giving rise to long (200 units) linear amylose chains, joined through 1 → 4 linkages. The reaction can be written as follows:

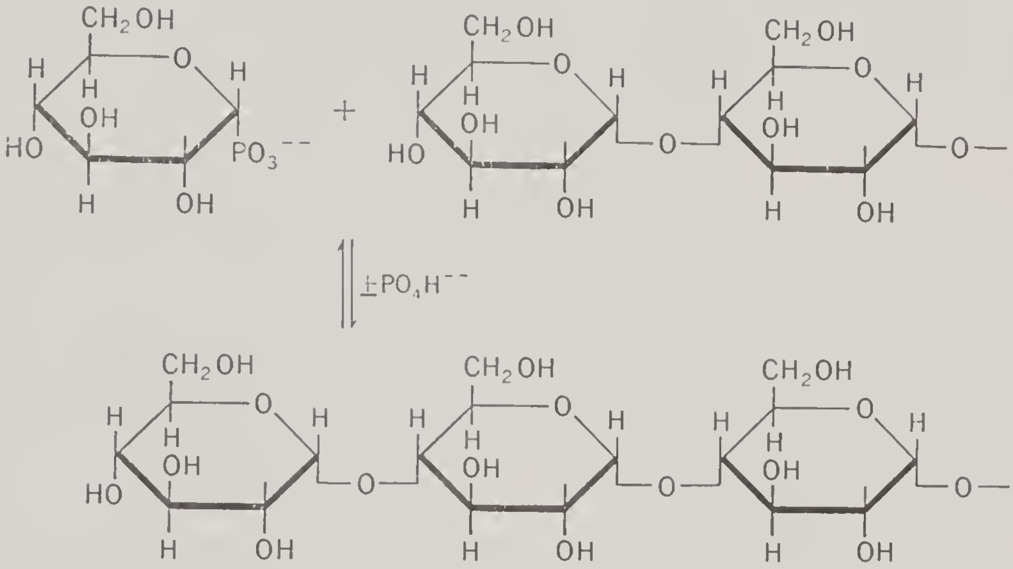


FIG. 4. The action of polysaccharide phosphorylase.

$$(C_6H_{10}O_5)_n + \text{Glucose-1-phosphate} \rightarrow (C_6H_{10}O_5)_{n+1} + P_i \qquad (67)$$

It is an interesting feature of Reaction 67 that the equilibrium does not depend, within wide limits, on the concentration of polysaccharide, because the concentration of nonreducing glucose ends does not change upon additions of new glucose units. On the other hand, the equilibrium varies with pH. The ratio of inorganic phosphate to ester phosphate varies from 10.8 to 3.1 in going from pH 5.0 to pH 7.0 (*225*).

Careful studies have been carried out on muscle phosphorylase. It can exist in two forms, a and b, of which only the latter has an absolute requirement for adenosine-5′-phosphate (*226*). The a to b transformation is catalyzed by another enzyme (PR-enzyme) and is accompanied by a halving of the molecular weight (*227*) and probably by a loss of protein-bound phosphate. Phosphorylase b can be reconverted into a by ATP, in the presence of a kinase (*228*).

Liver phosphorylase shows a somewhat different behavior. Although it can be attacked by a dephosphorylating enzyme, the protein formed has the same molecular weight as the intact enzyme, and it cannot be reactivated by adenylic acid (*229*). Here too, the active enzyme can be reconstituted by phosphorylation with ATP (*230*).

It has been found recently, that muscle phosphorylase a contains 8 moles of phosphate per mole, 4 of which belong to pyridoxal phosphate (*231*). It has also been possible to show that pyridoxal phosphate is a prosthetic group of phosphorylase a, essential for activity (*232*).

All these studies will undoubtedly lead to a clarification of factors which govern enzyme concentration within the cells, a point of the utmost interest in physiology.

(2) *Branching enzymes.* The amylose chains obtained through the action of phosphorylase are linear, while glycogen and amylopectin have highly branched structures. At the sites of branching the secondary chain is attached through a glucosidic group to the carbon-6 of a glucose belonging to the primary chain. The formation of the 1 → 6 linkages is catalyzed by a transglucosylase. It has been found in many animal tissues (*206*) and is usually called "the branching enzyme." It is also found in plants where it was called "the Q-enzyme." The Q-enzyme has been obtained in the crystalline form (*233*).

Branching enzymes act as transglucosylases, transferring a segment of a chain to position 6 of a glucose unit belonging to a different chain (*234*). This reaction seems to be practically irreversible, and this, together with other evidence (*144*), indicates that the free energy of hydrolysis of 1-6 linked glycosyls is significantly lower than that of 1-4 linked units.

By the combined action of phosphorylase and branching enzyme, substances with the same properties as glycogen and amylopectin have been synthesized *in vitro*.

(3) D-*Enzyme.* An enzyme which transfers glucose units has been isolated from potatoes (*234a,b,c*). Amylose-like polysaccharides can be formed from short-chain dextrins. The linkage is $\alpha(1 \rightarrow 4)$ both in the substrate and in the reaction product.

(4) *UDPG-glycogen transglucosylase.* An enzyme has been detected in mammalian tissues which catalyzes a transfer of glucose from UDPG to glycogen (*3a*) as follows:

$$\text{UDPG} + \text{Primer} \rightarrow \text{UDP} + \alpha(1 \rightarrow 4)\text{-Glucosyl-Primer} \qquad (67a)$$

The reaction resembles the phosphorylase reaction in several respects: the link formed is the same and a large molecular weight primer is required. On the other hand, the equilibrium of the reaction starting from UDPG is more favorable for synthesis than the one which starts with glucose-1-phosphate (see Section II,B).

There are some indications that in animal tissues glycogen is formed from UDPG and that phosphorylase may be mainly concerned with degradation (*3a*, *234d,e*).

b. Bacterial Glucans (*235*, *236*). Glycogen or glycogen-like polysaccharides can be formed by other mechanisms in which glucose-1-

phosphate is not involved. For instance, an enzyme (amylomaltase), which has been obtained from *E. coli* (*237*), catalyzes the reaction:

$$n(\text{Maltose}) \rightleftharpoons (\text{Glucose})_n + n(\text{Glucose}) \tag{68}$$

The glucan formed contains $\alpha(1 \rightarrow 4)$-linkages like glycogen (*238, 239*). In another mechanism for the formation of a glycogen-like polysaccharide, sucrose is used as substrate. With an enzyme from *Neisseria perflava* (amylosucrase), fructose is set free and a glucan is formed (*138*). It is believed that $\alpha(1 \rightarrow 4)$-linkages are first formed and that the chains are ramified by another enzyme just as in the case of glycogen.

Dextrans are high molecular weight polymers of glucose containing mainly $\alpha(1 \rightarrow 6)$-linkages with some branches joined by $\alpha(1 \rightarrow 4)$- or $\alpha(1 \rightarrow 3)$-linkages. Several bacteria such as *Leuconostoc mesenteroides, Betacoccus arabinosus,* etc., form dextrans from sucrose. The corresponding enzyme has been named dextran sucrase (*138*), and it catalyzes the transfer of glucose residues from sucrose with the liberation of fructose. The acceptor is a pre-existing dextran chain which becomes elongated. Since fructose has been detected in some dextrans, it is believed that the chains may be started by a molecule of sucrose acting as acceptor. Other sugars such as glucose or maltose can also act as acceptors when present in high concentrations (*240*).

Dextrans can also be formed from dextrins by the enzyme dextrin dextrinase of *Aerobacter capsulatum* (*241*). This reaction consists in a transfer of glucosyl groups from position 4 of one glucose residue to position 6 of another.

c. Cellulose. The most abundant carbohydrate in nature is cellulose, which is made up of β-glucopyranose units. Together with xylans, arabans, and lignin, it forms the walls of plant cells. The biosynthesis of cellulose has been studied both in plants (*129*) and in bacteria (*242, 243*) (*Acetobacter xylinum*), with the aid of C-1 or C-2 labeled glucose. Most of the label was found in the corresponding carbon atoms of the glucose units of cellulose. These results are consistent with the finding that extracts from *A. xylinum* catalyze the incorporation of glucose from UDPG into cellulose (*244, 244a*). As in the case of phosphorylase, a primer is needed; that is, no reaction takes place unless some chains are already present.

d. $\beta(1 \rightarrow 3)$-Glucans. Plant extracts catalyze the formation of $\beta(1 \rightarrow 3)$ glucans from UDPG. These glucans have a structure similar to that of callose which has been isolated from the phloem of some plants (*244b*).

2. *Synthesis of Fructans*

This process occurs mainly in plants, molds, yeasts, and bacteria. Many different fructofuranose-containing compounds are present in plants with various molecular weights ranging from 360 in sucrose to about 10,000. There are two types of compounds (*245*): the inulin and the phlein types. In inulin fructofuranose units are joined through the $\beta(2 \rightarrow 1)$-linkage, while in phlein they are joined through $\beta(2 \rightarrow 6)$. Fructans generally contain a small amount of glucose, as would be expected when polymerization takes place by successive addition of fructofuranose units to sucrose (*246, 247*).

Inulin is the reserve carbohydrate of Dahlia tubers and it is found in other members of the Compositae family such as Jerusalem artichoke. These plants contain numerous oligosaccharides with molecular weights reaching up to about 5000 for inulin (*248*).

The mechanism of synthesis of inulin is not well known, although some enzymes which are connected with it have been studied. Extracts of Jerusalem artichoke catalyze, not only hydrolysis, but also the transfer of fructose residues from and to inulin and associated polysaccharides. Sucrose can act both as a donor and as an acceptor of fructufuranose residues as follows:

$$\text{Sucrose} + \text{Sucrose} \rightarrow \text{Trisaccharide} + \text{Fructose} \qquad (69)$$

$$(\text{Fructose})_n + \text{Sucrose} \rightarrow \text{Trisaccharide} + (\text{Fructose})_{n-1} \qquad (70)$$

The essential difference between the enzyme from Jerusalem artichokes and invertases seems to be that the former is more active on higher molecular weight fructans. The transfructosylase is probably responsible for the interconversion of the numerous fructans found in the tubers of the artichoke. Fructans of the phlein type are found in many plants and especially in forage plants. They are formed by only 15–20 fructofuranose units with a terminal glucopyranose (*249*). Their role in plants seems to be that of a reserve material formed from sucrose (*250*).

Several bacteria (*Aerobacter levanicum, Bacillus subtilis, Bacillus mesentericus, Pseudomonas prunicola*, and *Leuconostoc mesenteroides*) contain an enzyme (levan sucrase) which catalyzes the formation of fructans from sucrose with the liberation of glucose. The enzyme is constitutive in some species and adaptive in others.

These bacterial fructans belong to the phlein type [$\beta(2 \rightarrow 6)$] of fructans, but their molecular weight is usually much higher than that of plant fructans. Some of the oligosaccharides produced as intermediates in levan synthesis contain a terminal glucose residue (*247, 251, 252*).

3. *Synthesis of Other Polysaccharides*

a. Chitin. Chitin, a polymer of acetylglucosamine, resembles cellulose in many properties. Both polysaccharides contain $\beta(1 \rightarrow 4)$-linkages and are very insoluble, a property which is convenient for a structural material. Chitin is present in molds, in the shells of crustacea, and in some microorganisms. Its synthesis has been studied with an enzyme preparation obtained from a mold (*Neurospora*). On adding UDP-acetylglucosamine labeled in the sugar moiety, the label was recovered in the chitin (*253*). The addition of a soluble (degraded) chitin preparation as a primer greatly increased the incorporation of label.

b. Mucopolysaccharides (*254–259*). The term "muco" is usually used to mean "amino group containing." Mucopolysaccharides are constituted by hexosamines and uronic acids. Only a few of them can be mentioned. Hyaluronic acid (acetylglucosamine, glucuronic acid) form the ground substance of connective tissue in animals. Chondroitin sulfate (acetylgalactosamine, glucuronic acid, and sulfate) is a constituent of cartilage.

Heparin, a natural anticoagulant, contains glucosamine and glucuronic acid and some nitrogen-linked sulfate. Many other compounds have been isolated from animal tissues, some of which contain L-iduronic acid, which is the 5-epimer of glucuronic acid (*260*).

Studies on the biosynthesis of hyaluronic acid by hemolytic streptococci have shown that the carbon chain of labeled glucose is converted to hexosamine and uronic acid residues without randomization (*256*). Studies have been also carried out with Rous sarcoma, which forms hyaluronic acid. Addition of labeled UDP-acetylglucosamine to cell homogenates led to the formation of labeled hyaluronic acid (*261*). Evidence was also obtained indicating that the glucuronic acid residue was transferred from UDP-glucuronic acid.

Studies with extracts of hemolytic streptococci have shown unequivocally that UDP-compounds are involved in the synthesis of hyaluronic acid (*261a*).

Whether the UDP compounds act as direct donors to polymer chains or whether oligosaccharides are formed first is a point which has not yet been settled. In this connection it may be mentioned that besides its hydrolytic action, hyaluronidase catalyzes transfer reactions, which may lead to rearrangements in the hyaluronic acid chains (*262*).

Derivatives of UDP are presumably involved in the synthesis of neuraminic acid polymers such as colominic acid found in *E. coli* (*42a*).

c. Bacterial Cell Wall Polysaccharides (*263*). Some UDP-hexosamine derivatives have been found to accumulate when *Staphylococcus* is treated with penicillin (*264*). The hexosamine in these compounds has turned out to be the same as one which has been isolated from the bacterial cell wall (*265*). It has been named muramic acid and is a derivative of acetylglucosamine. The substituent is lactic acid and the linkage is believed to be of the ether type between C-2 of the lactic acid and C-3 of acetylglucosamine (*266*). One of these UDP-muramic acids contains in addition several amino acids joined to the carboxyl group of the lactic acid rest. The cell walls were found to contain muramic acid and amino acids in the same ratio as they occur in the UDP compound. It has been suggested therefore (*43*) that the UDP compounds act as donors of building blocks for the formation of the bacterial wall and that the action of penicillin consists in inhibiting the transfer of peptide-muramic acid from UDP to the bacterial cell wall polysaccharide.

d. Polyuronides. Pectins are polymers of $\alpha(1 \rightarrow 4)$-linked galacturonic acid; they also contain arabans and galactans and are widely distributed in plants. Experiments on the formation of pectins in strawberry plants (*267*), have shown that radioactive hexoses are converted to polygalacturonides with only a small rearrangement of the carbon chain. This would be consistent with the following pathway:

Glucose → UDP-glucose → UDP-glucuronic acid → UDP-galacturonic acid → Pectin (71)

A pathway for polygalacturonide formation which has been suggested consists in the oxidation of galactan to polygalacturonide, that is, an oxidation at C-6 occurring directly on the polymer. However, this route has been excluded on the grounds that the linkages are $\alpha(1 \rightarrow 4)$ in galactans, while they are $\beta(1 \rightarrow 4)$ in polygalacturonic acids (*2*).

Another polyuronic acid found in nature is alginic acid. It is a polymannuronic acid found in algae. Nothing is known on its biosynthesis, but a compound is known, guanosine diphosphate mannose (*49*), which might well be involved in a series of reactions similar to that mentioned above.

e. Pentosans. Arabans and xylans are abundant in plants. Labeled glucose is known to be converted to xylan with only small rearrangement of the carbon skeleton and with loss of C-6 (*268, 129*). In these experiments it was found that glucuronolactone serves as well as glucose as a precursor of xylan while free xylose is transformed only after considerable rearrangement. Some of the possible pathways from glucuronic acid to pentoses have already been mentioned. The isolation of UDP-xylose and UDP-arabinose (*40*) may soon lead to more information on the synthesis of pentosans.

In fact, these studies have been already initiated with the finding that asparagus extracts catalyze the transfer of xylose units from UDP-xylose to low molecular weight $\beta(1 \rightarrow 4)$-xylose oligosaccharides (*269*).

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CHAPTER 3

Comparative Biochemistry of Free Energy Utilization for the Biosynthesis of Peptides and Proteins

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I. Introduction

For many years, it has been taken for granted that the synthesis of peptides and proteins is brought about by peptidases and proteolytic enzymes. An enzyme, being a true catalyst, is able to promote a reaction in both directions, and since the proteolytic enzymes were the only ones known to act upon peptide bonds, it seemed probable that they were responsible for amino acid condensation *in vivo*. It was assumed that the conditions in the living cell are sufficiently different from those obtained *in vitro* for the hydrolytic process to be reversed. Plastein formation *in vitro* and special cases of peptide synthesis catalyzed by proteolytic enzymes provided some support for this theory. However, it was untenable on thermodynamic grounds. The standard free energy of hydrolysis of the peptide bond of a dipeptide is about —3000 cal. This figure is a little less negative when the peptide chain is longer, but still of the order of —1000 cal. (*1–4*).

Taking into account the probable concentration of the amino acids in the cell, one realizes that the amount of dipeptide, for example, formed by reversal of hydrolysis will be very small (*3, 4*). The condensation of amino acids will not proceed to any appreciable extent unless it is driven by some process making energy available for the reaction.

The first observation showing the necessity of energy for peptide bond formation in the living cell was probably made by Krebs (*5*). Respiration or lactic fermentation were found to be necessary for the synthesis of glutamine in brain, retina, and kidney of rabbit and guinea pig. Borsook and Dubnoff (*6*) later showed that the amount of hippuric acid actually synthesized by rat liver slices *in vitro* is much greater than the amount expected if the condensation took place by reversal of hydrolysis. Up to one thousand times as much as the amount calculated for the equilibrium will pile up in disrupted cell preparations (*7*). No doubt the condensation of benzoate with glycine takes place only because energy is provided by some exergonic process. Borsook and Dubnoff (*6*) showed that deprivation of oxygen or poisoning with KCN immediately stops hippuric acid synthesis. This was direct evidence that this peptide bond formation was coupled with energy yielding reactions of the respiratory chain.

In a very similar way, Lipmann (*8*) showed that the acetylation of amines in liver homogenates depends on respiration, and that it is suppressed by dinitrophenol, an agent known to uncouple phosphorylation from respiration. This indicated that the energy required for peptide synthesis is funneled into the system through phosphorylation processes. With soluble enzymes extracted from pigeon liver, Lipmann (*8*) observed that adenosine triphosphate (ATP) is required and that it is used stoichiometrically, one mole of ATP being required for each mole of amine acetylated. The necessity for respiration was then easily explained by the ATP requirement of peptide synthesis, since ATP is regenerated by respiration.

The dependence of peptide synthesis on ATP has been confirmed for the formation of all the natural peptides studied so far: hippuric (*9*) and *p*-aminohippuric acids (*10, 11*), glutamine (*12, 13, 14*), glutathione (*15, 16*), asparagine (*17*), pantothenic acid (*18*), and taurocholic acid (*19*). The question as to how the pyrophosphate bond energy of ATP is used in peptide formation has been greatly clarified during the last few years by studies on animal, plants, and microorganisms.

II. Energy Coupling in the Synthesis of Small Peptides

A. SYNTHESIS OF PEPTIDES AT THE EXPENSE OF ENERGY-RICH COMPOUNDS

1. *Acetylation of Amines*

In his classical studies on the mechanism of amine acetylation using soluble preparations of pigeon liver enzymes, Lipmann and his group

(*20*) found that besides ATP a new cofactor was required, which was named "coenzyme A" (A for acetylation). Two enzymes are involved in the acetylating reaction, in pigeon liver preparations, and coenzyme A acts as a carrier of activated acetyl radical from one to the other (*21, 22, 23*). The real acetylating agent which reacts with the amine in the presence of one of the enzymes is acetyl-coenzyme A, a compound in which the acetyl radical is bound to the —SH group of coenzyme A through a thioester bond (*24, 25*). The condensation of the acetyl radical with the amine is a practically irreversible process. The driving energy for the condensation is provided by the rupture of the thioester bond of acetyl-CoA, whose energy content is roughly equal to that of the pyrophosphate bonds of ATP (*26, 27*).

$$\text{Acetyl} \sim \text{CoA} + \text{NH}_2\text{—R} \rightarrow \text{Acetyl—NH—R} + \text{CoA}$$

The problem of energy coupling in the acetylation of amines resides in the formation of the high-energy acetylating agent acetyl-CoA.

Acetyl-CoA can be formed by various processes according to the tissue or the organism considered. In pigeon liver and in yeast, the energy is provided by a pyrophosphate split of ATP (*28*). An isotopic study on the yeast enzyme system led Berg (*29*) to propose the following scheme:

$$\text{Acetate} + \text{ATP} = \text{Adenyl} \sim \text{acetate} + \text{PP}$$
$$\text{Adenyl} \sim \text{acetate} + \text{CoA} = \text{Acetyl} \sim \text{CoA} + \text{AMP}$$

Adenyl~acetate has not been isolated from the enzyme preparations; it does not accumulate in the medium and it must be admitted that the real intermediate is some form of adenyl~acetate strongly bound to the enzyme, and that the two reactions above are catalyzed by one and the same enzyme. The energy ultimately available for peptide formation in the thioester bond of acetyl-CoA originates from the second pyrophosphate bond of ATP.

In bacteria, acetyl-CoA can be formed from acetate at the expense of ATP by a completely different pathway. In *Escherichia coli,* for instance, ATP can phosphorylate acetate directly (*30*), forming acetyl ~phosphate

$$\text{ATP} + \text{Acetate} = \text{Acetyl} \sim \text{phosphate} + \text{ADP}$$

Acetyl phosphate, in contradistinction to adenyl acetate, is not strongly bound to the enzyme, and considerable amounts of the mixed anhydride pile up *in vitro.* Acetyl phosphate, in the presence of another well characterized enzyme, transacetylase (*22*), transfers the activated acetyl radical to coenzyme A

$$\text{Acetyl phosphate} + \text{CoA} = \text{Acetyl-CoA} + \text{P}_i$$

In the present case, the energy for the synthesis of acetyl-CoA is derived from the first bond of ATP.

Acetyl phosphate may be formed by aldehyde oxidation also (*31*), at the expense of the energy of oxidation

$$CH_3CHO + DPN^+ + PO_4 = \text{Acetyl phosphate} + DPNH + H^+$$

The oxidation of pyruvic acid (i.e., of carbohydrates) and of lipids (*32*) also produce acetyl-CoA. The energy thus stored in the thioester bond comes from the bond energy of the carbon chains and from oxidation reactions.

Finally, transacetylation reactions occurring between various acyl derivatives of CoA may funnel energy coming from many sources (*27, 33*) into acetyl-CoA.

The energy ultimately used for peptide bond synthesis in acetylation reaction may thus have many different origins. The high-energy reagent directly involved in the acetylation of the amine is always acetyl-CoA. This is one of the fundamental intermediary metabolites common to all living cells (*33a*).

The specificity of the acetylation reaction is not very well known. Liver enzymes are able to acetylate various aromatic amines, such as sulfanilamide, *p*-aminobenzoic acid, sulfadiazine, sulfathiazole, nitroaniline, etc. Glucosamine also is acetylated in pigeon liver by a similar system (*33b*). Enzymes have been found in *E. coli* which acetylate glutamic acid, aspartic acid, and a few more amino acids (*34*).

2. *Hippuric Acid Synthesis*

Energy coupling in hippuric acid synthesis resembles very much what has been found for the acetylation of amines. Coenzyme A is also involved (*35*) in this peptide bond formation. When the energy is provided by ATP, as in liver, it is the second pyrophosphate bond which reacts, probably by forming an enzyme-bound adenyl benzoate which then transfers the benzoyl radical to coenzyme A (*36, 37*).

$$\text{ATP} + \text{Benzoate} = \text{Adenyl benzoate} + \text{PP}$$
$$\text{Adenyl benzoate} + \text{CoA} = \text{Benzoyl-CoA} + \text{AMP}$$
$$\text{Benzoyl-CoA} + \text{Glycine} = \text{Hippuric acid} + \text{CoA}$$

It has been shown (*38*) that synthetic adenyl benzoate can indeed replace benzoate + ATP in this system. The first two reactions take place under the influence of a single enzyme, the "activating enzyme"; the third reaction is catalyzed by a "condensing enzyme," which is specific for the acceptor, glycine.

It seems rather probable that benzoylation of ornithine into ornithuric acid, which occurs in bird liver (*39*), follows a similar course, but that the condensing enzyme differs in its specificity towards the acceptor.

It has been found recently that benzoyl-CoA can arise by oxidation of the cyclohexane carboxyl-CoA (*40*). In this case, the thioester bond is formed before the aromatization, and there are indications that the process of activation is quite similar to benzoic acid activation by ATP.

What is known at present concerning *p*-aminohippuric acid synthesis (*10, 11*) resembles so closely hippuric acid synthesis, that it can be reasonably admitted that the two processes are essentially the same.

3. *Phenylacetylglutamine Synthesis*

The condensation of phenylacetic acid with glutamine seems to be peculiar to man and the chimpanzee. The enzymatic system has not been found in dog, rat, monkey, rabbit, horse, sheep, or cat (*37, 38*).

The principle of energy coupling is the same here as for hippuric acid synthesis. It is remarkable that the enzyme which activates phenylacetic acid as enzyme-bound adenyl phenylacetate and which transfers the activated radical to CoA is found in beef liver, but that the condensation with glutamine does not occur in this case. It is the condensing enzyme specific for glutamine which is characteristic of man and the chimpanzee while the activating enzyme is present in other mammals.

4. *Taurocholic Acid Synthesis*

Elliot (*19*) has shown that taurocholic acid results from the condensation of taurine with cholyl-CoA. The coupling is thus most probably of the same type as in the preceding cases.

5. *Pantothenic Acid Formation*

Pantothenic acid synthesis has been observed in *Escherichia coli* (*18*), *Neurospora* sp. (*41*), and in yeast (*42*). Maas and Novelli (*43*) have shown that in *E. coli* extracts coenzyme A is not required. ATP is used stoichiometrically, and it is split into AMP and pyrophosphate (PP). The energy is again provided by the second bond of ATP and the reaction sequence seems to be

$$\text{ATP} + \text{Enzyme} + \text{Pantoic acid} = \text{Enzyme-adenyl pantoate} + \text{PP}$$
$$\text{Enzyme-adenyl pantoate} + \beta\text{-Alanine} = \text{Pantothenic acid} + \text{Enzyme} + \text{AMP}$$

The activation of the carboxyl group and its condensation with the amine are both catalyzed by the same enzyme.

6. *Glutamine Synthesis*

Speck (*12*) studied the synthesis of glutamine from glutamic acid and ammonia, in pigeon liver extracts, and showed that the ATP requirement is stoichiometric. One mole of ATP is split into ADP and inorganic phosphate (P_i) for each mole of glutamine synthesized.

$$\text{Glutamic acid} + NH_3 + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + P_i$$

The carboxyl of glutamic acid is probably activated, for in the presence of hydroxylamine the corresponding hydroxamic acid is formed. Later, Elliot (*44, 45*) made similar observations with enzymes purified from sheep brain, and he found enzymatic systems forming glutamine in rabbit kidney, in the meal worm, and in *Lupinus* seedlings; the enzyme is also present in several microorganisms (*45, 46*) and in green peas (*47, 48*).

In spite of many attempts by several workers, no soluble glutamic acid derivative with an activated carboxyl have been isolated (*47*) and it must, at present, be concluded that the activated compound is probably bound to the enzyme. This is supported by the fact that one single enzyme is involved and that both the activation and the condensation take place on its surface.

A bound glutamyl phosphate intermediate is postulated (*48–50*) because inorganic phosphate does not exchange with the terminal phosphate groups in ATP unless glutamic acid and ammonia both are present. No free glutamyl phosphate has been detected and synthetic glutamyl phosphate cannot replace ATP plus glutamate. However, the carboxyl group of glutamic acid must at some time be bound to phosphate, or be replacing phosphate on the enzyme surface, for glutamic acid labeled with heavy oxygen on the γ-carboxyl group transfers the isotope stoichiometrically to inorganic phosphate and not at all to the ADP moiety of ATP.

$$\text{Enzyme} + \text{ATP} + \text{Glutamic acid} \rightleftharpoons (\gamma\text{-Glu} \sim \text{P})\text{Enzyme} + \text{ADP}$$
$$(\gamma\text{-Glu} \sim \text{P})\text{Enzyme} + NH_3 \rightarrow \gamma\text{-Glutamine} + P_i + \text{Enzyme}$$

The detailed working of the enzyme is not yet clear, but the above scheme accounts satisfactorily for the presently known facts.

7. *Formation of Glutathione*

Glutathione, γ-glutamylcysteinylglycine, contains two peptide bonds. Both are formed by a very similar mechanism. Each depends on the splitting of one molecule of ATP into ADP and phosphate (*15, 16*). Coenzyme A is not involved in the process (*15, 50*).

The main features of glutathione synthesis have been established with liver (*16*) and bean (*15*) extracts. However, the detailed study of the energy coupling has been made with highly purified enzymes from yeast (*51*) and from wheat seedlings (*50, 52*).

The first bond to be formed, in plants as well as in animals, is that of γ-glutamylcysteine. This condensation very much resembles glutamine formation. The exchange reactions catalyzed by this enzyme are quite similar to those brought about by glutamine synthetase, except that cysteine here plays the part of ammonia in the other system.

γ-Glutamylcysteine then condenses with glycine under the action of a second enzyme. Here again the exchange reactions are of the same type, and the mechanism of the energy coupling can be represented as follows (*53*):

$$\begin{gathered}
\mathrm{Enz_1 + ATP + Glu \rightleftharpoons (\gamma\text{-}Glu \sim P)Enz_1 + ADP} \\
\mathrm{(\gamma\text{-}Glu \sim P)Enz_1 + Cys \rightarrow \gamma\text{-}Glu\text{-}Cys + P_i + Enz_1} \\
\mathrm{Enz_2 + ATP + \gamma\text{-}Glu\text{-}Cys \rightleftharpoons (\gamma\text{-}Glu\text{-}Cys \sim P)Enz_2 + ADP} \\
\mathrm{(\gamma\text{-}Glu\text{-}Cys \sim P)Enz_2 + Gly \rightarrow Glutathione + P_i + Enz_2}
\end{gathered}$$

8. *Other Peptides*

The mechanism of carnosine and anserine synthesis has, as yet, not been studied in detail. The existence of β-alanyl-CoA has, however, been established in certain microorganisms (*54*), where it is formed from acrylyl-CoA by fixation of NH_3 on the double bond. It is thus quite possible, although not certain, that both β-alanyl peptides are formed by a mechanism similar to hippuric acid synthesis.

The participation of coenzyme A in the condensation of hemin with protein (*55*) makes one suspect that a CoA activation of the carboxyl groups of hemin might possibly be involved.

In the chain of reactions leading to purine biosynthesis, a peptide bond is formed between glycine and phosphoribosylamine. This step also has been shown to depend on ATP, but its mechanism has not yet been studied; a splitting of ATP between the ADP moiety and the terminal phosphate group is indicated (*56*).

The synthesis of coenzyme A itself seems to proceed via condensation of pantothenic acid with cysteine, forming pantothenylcysteine. This peptide bond formation, which takes place in rat liver, for example, is also coupled to ATP splitting (*57*).

9. *Basic Features of Energy Coupling in Peptide Synthesis*

The comparison of the different cases of peptide synthesis allows the following rules to be derived:

In all the cases considered, the formation of peptide bonds requires the activation of the carboxyl group involved.

The energy is generally derived from a pyrophosphate bond of ATP. Two types of splitting of ATP may be distinguished.

a. Pyrophosphate Splitting of ATP. When the bond between the AMP moiety and the terminal pyrophosphate of ATP is used, the first product of the reaction is a mixed anhydride of AMP and the carboxyl group of the acid involved. This activated form of the acid is strongly bound to the enzyme.

The activated carboxyl may react directly with the acceptor amine on the enzyme where it was formed, as in the case of pantothenic acid synthesis. In most cases, however, the activated carboxyl is transferred to coenzyme A, forming an acyl-CoA which is the real acylating agent. The acyl-CoA will react with the acceptor at the level of another enzyme, generally specific for the acceptor, in forming the peptide.

When the transfer of the activated carboxyl group is mediated by coenzyme A, the energy in the thioester bond is not necessarily derived from ATP. Various acyls are bound to coenzyme A in many oxidations and in the controlled breakdown of aliphatic chains. The energy is thus directly stored in the thioester bond of an acyl-CoA, and various coenzyme A transacylases make possible a redistribution of the available energy between several acyl radicals.

b. Phosphate Splitting of ATP. When the bond between the ADP moiety and the terminal phosphate group of ATP is providing the energy, as in γ-glutamine synthesis, the activation most probably again concerns the carboxyl group. In this case both the activation and the condensation occur on the same enzyme.

The fundamental principle of the coupling is the same in all cases. One of the components of the original high-energy bond, of ATP for instance, is replaced by another radical of such a nature that there is little energy change in the process (K not very different from 1). In this way, the energy content of the bond is preserved, although the nature of the bond has changed. After a few such replacements, the available energy is found in a compound containing no part of the original high-energy compound, but retaining most of the energy that was there in a bond involving one of the radicals to be condensed. For example:

$$\text{A-5}'\text{-P} \sim \text{P} \sim \text{P} + \text{CH}_3\text{COOH} = \text{A-5}'\text{-P} \sim \text{CO—CH}_3 + \text{PP}$$
$$\text{A-5}'\text{-P} \sim \text{CO—CH}_3 + \text{CoA} = \text{CH}_3\text{CO} \sim \text{CoA} + \text{A-5}'\text{-P}$$

The high energy bond involving the carboxyl will then react with the NH_2 group of the acceptor:

$$\text{CH}_3\text{—CO} \sim \text{CoA} + \text{R—NH}_2 \rightarrow \text{CH}_3\text{—CO—NH—R} + \text{CoA}$$

In this final reaction, part of the energy will be used for making the peptide linkage, and a rather large part will be dissipated, thus making the condensation irreversible for all practical purpose and the reaction complete.

The synthesis of each type of peptide has been studied in very few organisms or tissues, and the details of the coupling have generally been worked out only once in a single material for each peptide. However, in many cases, the general properties of the system has been shown to be the same in mammals, birds, higher plants, yeast, and bacteria. It seems that each peptide is built up in quite the same way in all kinds of cells. The process of activation and energy coupling, in all instances, is the same.

When differences among organisms have been observed, they appear to concern the condensing enzyme, i.e., the last stage of the process, and not the process of activation and energy coupling. Both mammal and bird liver activate benzoic acid and form benzoyl-CoA. Mammals condense it to glycine, birds to ornithine. Various mammals are able to activate phenylacetic acid, but only man and the chimpanzee condense it with glutamine. Bacteria and mammals make acetyl-CoA. Mammals condense it with aromatic amines while bacteria cause it to react with glutamic and aspartic acids.

B. Formation of Peptides by Transpeptidation

Many cases of peptide formation by transpeptidation have been reported since the first observations by Bergmann (*58, 59, 60*).

1. *Transglutamylation*

a. From Glutamine. In extracts of *Proteus vulgaris* (*61, 62*), enzymes have been found which catalyze the exchange of the NH_2 group of γ-glutamine or β-asparagine for free ammonium ions, or the replacement of NH_2 by hydroxylamine. Sheep liver, brain, and kidney, mouse liver and sarcoma contain a similar system reacting in the same way with glutamine (*63*). An enzyme named glutamyl transphorase or glutamyl transferase has been isolated from pumpkin seedlings (*64–66*); it catalyzes the same exchange or replacement reactions in glutamine. Manganese ions activate the transglutamylation in plant and animal tissues, but not in bacterial extracts (*67*). With the pumpkin enzyme, phosphate or arsenate, and traces of ATP or ADP are required (*65*). It is important to realize that only catalytic amounts of ATP or ADP are necessary for these transglutamylations. Their function is not to provide energy, as in the peptide synthesis studied previously where stoichiometric amounts of ATP were split during the reaction. In the present system, ATP or ADP act as cofactors.

In plant and animal tissues, glutamyl transphorase seems to be identical to the enzyme glutamine synthetase, or closely associated with it. No separation of the two systems was achieved in pigeon liver extracts (*67, 68*). A 1000-fold purification of the enzyme in pea extracts also failed to separate the two activities (*47*). On the other hand, microbial glutamotransferase, which does not require Mn or ATP, can be completely freed from synthetase activity (*69*). During growth of the chick embryo, moreover, the ratios of the two activities showed some variation (*67*).

b. From Glutamyl Peptides, Especially Glutathione. The formation of a variety of γ-glutamyl peptides by transglutamylation from glutathione to amino acids and peptides occurs in extracts of mammalian kidney and pancreas (*70–73*).

$$\text{Glutathione} + \text{Valine} = \gamma\text{-Glutamylvaline} + \text{Cysteinylglycine}$$

Glutathione is not a unique donor in this system; the enzyme catalyzes the transfer of γ-glutamyl group from a range of γ-glutamyl peptides to a series of acceptors. The enzyme involved resembles a hydrolase specific for γ-glutamyl peptides, and hydrolysis occurs concurrently with transacylation.

2. *Transpeptidation from Various Amides*

Quite a number of transpeptidation reactions catalyzed by proteolytic enzymes have been reported and extensively studied during the last few years. Apart from several types of transpeptidation (*74, 75*) the transfer of the acyl radical from an amide to an amino acid or a peptide, with elongation of the peptidic chain, are especially interesting examples of transpeptidation.

Such reactions take place *in vitro* in the presence of the following purified enzymes from plant or animal origin: papain, ficin, trypsin, several cathepsins, and chymotrypsin (*76–76d*).

For instance, beef spleen cathepsin catalyzes the following types of reactions with glycylphenylalanylamide (*76*):

$$\text{Gly-Phe-NH}_2 + \text{N}^{15}\text{H}_3 \rightleftharpoons \text{Gly-Phe-N}^{15}\text{H}_2 + \text{NH}_3$$
$$\text{Gly-Phe-NH}_2 + \text{NH}_2\text{OH} \rightleftharpoons \text{Gly-Phe-NHOH} + \text{NH}_3$$
$$\text{Gly-Phe-NH}_2 + \text{Gly-Phe-NH}_2 \rightleftharpoons \text{Gly-Phe-Gly-Phe-NH}_2 + \text{NH}_3$$

The last reaction can be repeated and the stepwise additions of the Gly-Phe moiety of glycylphenylalanine amide to the peptide formed at the preceding step results in the formation of octa- or decapeptides composed of 4 or 5 Gly-Phe units; these peptides are rather insoluble and tend to come out of solution.

3. *Acyl Transfer from Esters of Amino Acids*

Proteolytic enzymes are not quite specific for the peptide bond. Several of them can cause the hydrolysis of esters (*77*). These enzymes can also transfer the acyl radical from amino acid esters to amino acid and peptides, thus leading to the formation of new peptide bonds (*78–81*).

4. *Energy Relationships in Transpeptidations*

In transpeptidation reactions no additional peptide bond is created. What is observed is the replacement of a peptide bond between an acyl group and an amino group by a peptide bond between the original acyl radical and another amine. All the transpeptidations which have been studied so far seem to involve the transfer of an acyl radical, but no transfer of an amino group has been reported.

When a peptidic bond is replaced by another one having almost identical properties there is no change in the standard free energy; the reaction proceeds in one or the other direction depending on the concentrations of the reactants. There is no need for exogeneous energy for exchanging a $N^{14}H_2$ group for the same group labeled with N^{15}. The energy change is probably very low when hydroxylamine replaces ammonia.

In transglutamylation from glutathione, it may be that the reaction is favored by the splitting of cysteinylglycine under the action of a very active dipeptidase present in the same kidney extracts as the transferring enzyme.

$$\gamma\text{-Glu-Cys-Gly} + \text{Val} \rightleftharpoons \gamma\text{-Glu-Val} + \text{Cys-Gly}$$
$$\text{Cys-Gly} + H_2O \rightarrow \text{Cys} + \text{Gly}$$

The splitting of cysteinyl-glycine makes the transglutamylation go to completion by removing one of the products of the reaction. This is a kind of energy coupling (*60*). It is the irreversible hydrolysis of the dipeptide which displaces the equilibrium of the first reaction to the right. In the process two peptide bonds of glutathione disappear and one new bond is formed. Part of the energy used in the making of the latter is derived from both peptide bonds of glutathione.

A comparable coupling may also be observed when the low solubility of one of the resulting peptides prevents the equilibrium of the transpeptidation from becoming established and thus allows the reaction to go to completion. These types of coupling are rather ineffective when compared to the direct stoichiometric coupling observed in reactions deriving their energy from ATP or other high energy bonds.

Transacylation from amides or from esters may represent an intermediate situation. It is possible that the standard free energy of hydrolysis of these bonds is somewhat more negative than that of the typical peptide bond formed. Amides and esters may then represent higher energy bonds, although at a level much lower than those of ATP, acyl anhydrides, or thioesters.

In the transacylations catalyzed by proteolytic enzymes, it may be visualized that the acyl radical is bound to the enzyme and that the acyl-enzyme complex can be attacked by various acceptors, i.e., NH_2—R, NH_3, NH_2OH, and H_2O, forming respectively a peptide, an amide, a hydroxamate, or the free acid (*82*). The mechanism does not differ essentially from the transfers between higher energy compounds except for the factor of competition with water, which seems to be more important. This factor can, however, be increased or decreased according to the experimental conditions (*83, 84*). Glutamyl transfer from glutathione or other peptides seem to fall into the same category as the transacylations catalyzed by proteolytic enzymes.

In glutamotransferase exchanging the NH_2 of glutamine for NH_2OH, water seems not to compete with the amines. Glutamotransferase activity, however, may be without much physiological meaning. It seems to reflect a property of glutamine synthetase, just as the exchange of pyrophosphate with the terminal group of ATP is a property of the pantothenic synthesizing enzyme, and is of no interest as far as ATP synthesis in the cell is concerned.

The transpeptidation reactions leading to the synthesis of new peptides from preformed peptides, from amides, or from esters have been studied with purified enzymes isolated from plants, animals, and bacteria. Their physiological significance at present is not clear. In these reactions there is no great problem of energy coupling.

III. Energy Coupling in Protein Biosynthesis

A. Energy Requirement for Protein Synthesis

The first evidence that protein synthesis depends on energy-yielding reactions was provided by studies using the tracer method. It was observed that the incorporation of labeled amino acids into proteins of tissue slices (*85–88*) or of homogenates (*89–91*) requires the respiration of the system. Frantz and Zamecnik (*88*) clearly established that amino acid incorporation depends on phosphorylation by showing that the inhibition of the process by dinitrophenol is parallel to the inhibition of phosphorylation, and not to the rate of respiration *per se*. In homogenates, ATP or systems able to regenerate it are required for protein synthesis (*89–91*). Although this coupling of phosphorylation with protein synthesis has been known for several years, the mechanism of the coupling has not been completely elucidated.

One of the possibilities considered was that oligopeptides, like glutathione, are first formed by systems deriving energy from ATP, and that the long polypeptide chains of the proteins are then formed through a series of transpeptidations (*83, 92, 93*). Glutathione, glutamine, and glutamyl peptides have received special attention as possible initiators of protein formation (*70, 71*). However, no convincing evidence has been presented for the participation of these or other peptides. Direct attempts (*94, 95*) to test this idea have led to negative conclusions, and the bulk of the present data speak against such a mechanism for funneling energy into protein synthesis. If peptides were formed by a series of transpeptidations at the energy level of the peptide bond, the concentration of intermediary peptides would be rather high. And in spite of many attempts, no intermediate peptides have been found to accumulate in living cells. It is true that several observations on the nonuniform labeling of proteins in tissue slices (*96–98*) may be interpreted by postulating the existence of a pool of peptide precursors. But other interpretations of these data also are possible, and they cannot be taken as a definite proof of the participation of peptide intermediates (*99, 100*).

On the contrary, very convincing data have been obtained which show that if such a pool of intermediate peptides exists, it is very small. In the case of induced enzyme formation in microorganisms, proteins are formed directly from amino acids and an upper limit has been determined for the amounts of intermediates of any nature between amino acids and protein. This limit is very low and actually set by the sensitivity of the tracer method used, and the specificity of the analytical methods (*101–104*). Milk proteins (*105, 106*), γ-globulins (*106–108*), proteins of the liver (*109, 110*) or pancreas (*111*), Bence-Jones proteins (*112*) also are formed from free amino acids. All this makes it most probable that small peptides do not play any part in protein synthesis and that the energy coupling for protein synthesis is independent of the systems involved in the formation of the small natural peptides. It is reasonable then to look for systems activating the individual amino acids, similar to those known to activate acetate, benzoate, pantothenate, etc. (*108*).

B. Amino Acid Activation

Extracts obtained from rat liver (*113, 114*), pancreas (*115–117*), mammary gland (*118*), chicken erythrocytes (*119*), bone marrow (*119*), sea urchin eggs (*120*), yeast (*121*), and bacteria (*119, 122–125*) catalyze the exchange of the two terminal groups of ATP for pyrophosphate, in the presence of several amino acids. This is similar to the exchange observed with other enzymes in the presence of acetate, benzoate, or pantothenate. As we have seen before, this type of reaction indicates the formation of acyl-adenylate compounds (AA), and may be visualized in the following way:

$$\text{Enzyme} + \text{ATP} + \text{AA} = \text{A-5'-P-AA Enz} + \text{PP}$$

This reaction scheme has received confirmation from studies on the exchange of heavy oxygen between the carboxyl of the amino acid involved and the AMP moiety of ATP (*126*). Synthetic leucyl adenylate in the presence of the enzyme and of pyrophosphate forms ATP and leucine (*122, 123*). If high concentrations of hydroxylamine are present, the acyl group is trapped and the corresponding hydroxamic acid is formed.

An enzyme which activates tryptophan specifically has been isolated and crystallized from beef pancreas (*115*) while an enzyme which activates methionine has been purified from yeast (*121*). Enzymes specific for threonine and serine have been found in pancreas (*116*), and tyrosine is activated by purified extracts from yeast (*127*) and hog pancreas (*117*). For some time, it seemed that only about half of the natural amino acids were activated in this way. However, recent reports (*125, 128–130*) indicate that all the amino acids can be activated on the carboxyl by extracts from plants, animal tissue, and bacteria (see Table I).

The existence and the general occurrence of enzymes of this type in all kind of organisms is no evidence of their participation in protein synthesis. There are, however, good reasons to believe that these reactions represent the first stage of protein synthesis. In liver homogenates, the incorporation of amino acids into microsomal proteins requires the presence of a fraction which is known to contain the activating enzymes (*131–134*). It has been found also that the tryptophan-activating enzyme from pancreas, which does not activate any other natural amino acid, does activate several analogues of tryptophan. These analogues can be incorporated into proteins instead of tryptophan. On the other hand the enzyme does not activate several tryptophan analogues which are not incorporated (*115*). This parallelism between the specificity of the enzyme and that of protein synthesis makes it very probable that the activating enzyme is indeed involved in the process.

The amino acyl adenylates, like acetyl adenylate or benzoyl adenylate, are strongly bound to the enzyme where they are formed. One then wonders how these activated amino acids are transferred to the site of their condensation, the microsomes. Coenzyme A, which is the carrier of several activated acyl radicals, does not seem to carry amino acyl groups (*135*). But the crude preparations of activating enzymes contain a low molecular weight ribonucleic acid, to which the amino acyl radicals are transferred (*131, 136–138*). From there they are incorporated into the microsomes by a process, mediated by guanosinetriphosphate, which is still completely obscure (*130, 139*). More recently, the participation of vitamin B_{12} also has been indicated (*140*).

Recent studies on microorganisms may throw light on the relation between activated amino acids and oligonucleotides. It has been found that ribonucleic acid synthesis in bacteria and yeast (*141–145*) requires the simultaneous presence of all the amino acids, even when no protein synthesis is taking place. This suggests that ribonucleic acids and proteins are formed from a pool of common precursors, which are visualized as amino acids bound to oligonucleotides (*142–144, 146, 147*). These compounds might give alternatively polynucleotides plus free amino acids, or proteins plus oligonucleotides.

The inhibition of protein synthesis by ribonuclease (*148*) may be due to the destruction by the enzyme of these oligonucleotide carriers of activated amino acids, or to a modification of the nucleic acid templates.

In the present state of our knowledge, the most probable scheme of energy transfer for protein synthesis seems to be the following: The individual amino acids are probably activated by pyrophosphate split of ATP in the same way as are acetic, benzoic, or pantothenic acids. The activated amino acyl radicals are transferred to some oligonucleotidic compounds in a reaction at the level of the high-energy bonds, without much change of free energy. Finally, the amino acids, directed by their oligonucleotide carriers, find the correct place on a polynucleotidic template (*149*) and they condense there into a polypeptide. This last step is probably accompanied by the dissipation of a large part of the energy of the bond, and it is irreversible for all practical purposes.

C. SUMMARY AND CONCLUSION

At this time, when a picture of protein synthesis is beginning to emerge, it is impossible to go deeply into a comparison of the processes by which proteins are formed in different organisms. No systematic studies have been made for the purpose of such a comparison. It cannot be excluded *a priori* that certain cells may have developed special systems for the utilization of peptides or for partial transformation of proteins made by other cells. However, the general rule is certainly that

TABLE I
Amino Acid Activation

Organism and Tissue	Activated amino acids[a]	Reference
Rat		
Liver	Mixture	*113–115, 116, 130a*
	Phe, His, Try, Val, Ile, Cys, Met, Tyr, Leu	*119*
Kidney	Mixture	*116, 119*
Pancreas	Mixture	*116*
Brain	Try, Phe, Gly, Val, Leu, Cys	*130b*
Uterus	Thr, Leu, Tyr, Val, Met, Gly, Ile	*130c*
Heart	Mixture	*119*
Spleen	Mixture	*119*
Bone Marrow	Mixture	*119*
Guinea pig		
Liver	Mixture	*116*
Kidney	Mixture	*116*
Pancreas	Ser, Thr, Try.	*116*
Muscle	Mixture	*116*
Gut	Mixture	*116*
Mammary gland	Mixture	*116*
Rabbit		
Liver	Mixture	*116*
Kidney	Mixture	*116*
Muscle	Mixture	*116*
Ox		
Pancreas	Try and Try-analogs	*115, 130d*
Mammary gland	Mixture	*118*
Pig		
Liver	Phe	*130e*
	Ala	*130f*
Pancreas	Tyr, Try	*117*
Chicken		
Liver	Mixture	*116*
Kidney	Mixture	*116*
Sea urchin		
Unfertilized egg	Gly, Mixture	*120*
Plants		
Pea seedling	Leu, Tyr, Val, Try, Ile, Cys, Met	*130g*
	Leu, Tyr, Val, Try, Ile, Cys, Met, Phe, Ala, Arg, His	*130*
Carrot root	Mixture	*130g*
Spinach leaf	All except Ser	*130h*

TABLE I (*Continued*)

Microorganisms		
Yeast	Met	*121*
	Tyr	*127, 130i*
	Mixture	*125*
Neurospora crassa	Ala, Asp, AspNH$_2$, Glu, Ser, Thr, Try, His, Phe, Tyr, Val, Leu, Ile, Cys	*119*
Escherichia coli	Try, His, Phe, Met, Tyr, Val, Leu, Ile	*119, 122, 123*
	All the amino acids	*130j*
Aerobacter aerogenes	Phe, Met, Tyr, Val, Leu, Ile, Cys, Ala	*119*
Micrococcus lactilycticus	Ser, Try, Met, Tyr, Val, Leu, Ile, Cys	*119*
Desulfovibrio desulfuricans	Mixture	*119, 122, 123*
Rhodospirillum rubrum		
Leuconostoc mesenteroides		
Azotobacter vinelandii		
Clostridium butylicum		
Proteus vulgaris		
Clostridium pasteurianum		
Staphylococcus aureus		
Serratia marcescens		
Streptococcus hemolyticus		

[a] The amino acids mentioned in the table are those which have been shown to be activated. In most cases, all the natural amino acids have not been assayed. "Mixture" means that a mixture of several amino acids has given a positive reaction in the activation experiments, but that the individual amino acids have not been assayed separately.

proteins are formed from free amino acids which must be activated before they can condense into polypeptides. Many observations on this initial step in protein formation which were made on animal cells have been easily repeated and confirmed with plants and microorganisms, and *vice versa*. Therefore it is clear already that the same fundamental mechanism for protein synthesis operates in all living systems. Phosphorylation provides the energy and ATP delivers it. Amino acids are activated on their carboxyl group and the energy is probably stored first in an anhydride bond.

It is striking, although not unexpected, to find similarity in the fundamental mechanism of the very processes in which biochemical diversity most manifests itself. For diversity in the proteins is the basis of metabolic diversity and to a certain extent of morphological diversity. It is also the simplest expression of differences in the genetic material; a modification in the fine structure of a protein is indeed the most elementary change resulting from a gene mutation which can be detected at present. Thus, the diversity of proteins does not depend on differences in details of the biochemical processes by which building blocks and energy are provided, but is a direct expression of diversity in the genetic material.

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CHAPTER 4

Ammonia Metabolism and Urea Biosynthesis

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I. Introductory Remarks

The enzymatic systems catalyzing the formation of ammonia from amino compounds, amides, etc., are widely distributed. In general, plants, microorganisms, and animals, as far as they have been studied, have many if not all of the typical enzymes. Plants and microorganisms, however, are distinct from animals in that the former can perform extensive manipulations upon inorganic nitrogen compounds other than just ammonia.

For the purpose of the present review, which is of ammonia metabolism, discussion of the ammonia producing systems will be limited to the main types of reactions, and very brief reference will be made to their distribution, properties, etc. Because a number of comprehensive reviews dealing with the distribution, substrate specificity, purification, coenzyme requirements, etc., of the enzyme systems under discussion have appeared in recent years, no attempt will be made to deal with this aspect comprehensively.

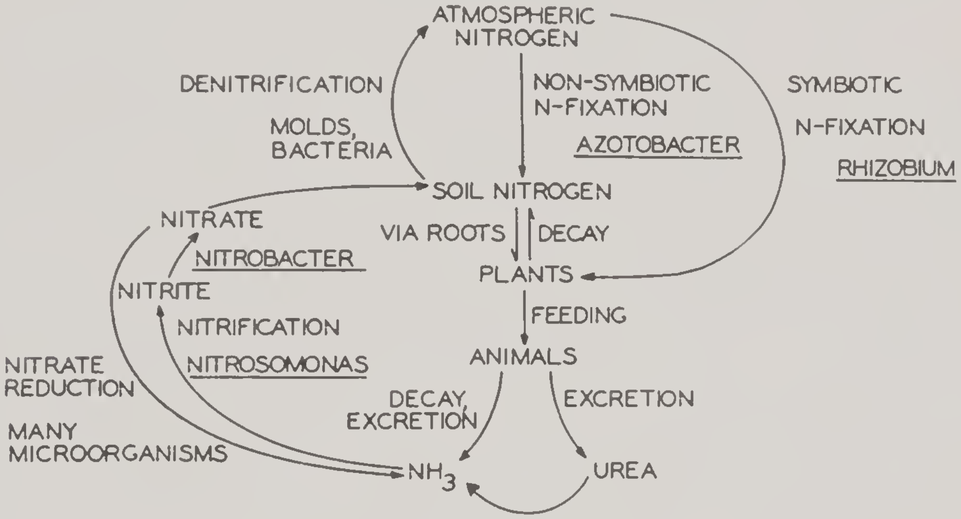

Fig. 1. Abbreviated nitrogen cycle.

In general, only those reactions involved more or less directly in ammonia production and utilization will be considered. Selection of references is based also in part on preference for studies in which extracts and purified preparations have been used rather than whole cells or intact organisms.

Since anabolic and catabolic pathways are often conditioned by the magnitude of free energy changes for individual reactions, discussion of the energetics of various systems is included.

Because of the interest from a comparative standpoint of the mechanisms of nitrogen excretion, the urea cycle (ornithine cycle) is treated extensively. The discussion of the thermodynamics of urea biosynthesis has been reserved for a later section, for this cannot be correlated properly with the general material of the chapter until appropriate enzymes and reactions have been discussed.

Ecological considerations of ammonia metabolism are beyond the scope of the present chapter. But to orient the reader, a highly abridged biological nitrogen cycle is depicted in Fig. 1. As is evident from the scheme, many types of living forms participate in the maintenance of the cycle, or more appropriately, of the network. In view of the fact that nitrogen is an essential element of protoplasm, it is clear that a close interdependency exists among *all* organisms as regards the metabolism of nitrogen compounds, and more specifically, that of ammonia.

II. Thermodynamic Data and Conventions

Much of the thermodynamic data employed in this chapter is present in the literature, and we have drawn freely from such works (*1–5a*). Values for the standard free energy changes in the chemical literature are based on a reference temperature of 298°K (United States). Standard free energy changes obtained from biochemical determinations of equilibrium constants usually have a reference temperature of 310°K or 311°K. The form of such equilibrium constants usually is such as to represent the total of the various ionic species of a given compound.

The notation, ΔF° $(= \Delta G^\circ)$, is employed for the standard free energy change.* If the free energy change has a negative value the reaction is spontaneous. If no temperature is indicated by subscript, the value of the free energy change is that assumed for either 298°K or 310°K. For our purposes little error is introduced if the values at the various reference temperatures are set equal. The notation, $\Delta F^{\circ\prime}$, is employed for the free energy change at pH 7 with all reactants and products except H^+ at unit thermodynamic activity. The free energy change under other specified conditions is ΔF. For our purposes it is sufficient to assume that activity equals molar concentration.

Considerable error can be introduced in calculating the value of ΔF° or $\Delta F^{\circ\prime}$ for a given reaction if the appropriate ionic species which actually react are not employed. Since in many cases the reacting species are not known, the predominant species present at the given reaction pH are employed for calculation. Failure to use appropriate ionic species in calculating free energy changes from standard free energies of formation can lead to a difference of as much as 1.419 (pK_{eq}) kcal./mole at 310°K.

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* The following abbreviations are used in this chapter: DPN^+ and DPNH, diphosphopyridine nucleotide and its reduced form; TPN^+ and TPNH, triphosphopyridine nucleotide and its reduced form; FAD and FAD•2H, flavin adenine dinucleotide and its reduced form; FMN and FMN•2H, flavin mononucleotide and its reduced form; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; K_m, Michaelis constant; R, gas constant; T, temperature; F°, H°, S°, free energy, heat content (enthalpy), and entropy, respectively, in the standard state (298° K)—subscripts are used to denote reference temperatures (see also Section II); P, pressure; ~, energy-rich bond; superscript numbers given with a compound denote the ionic species used in establishing or calculating the associated free energy datum, except that charges on the phosphate(s) of nucleotides are not so shown.

The activity of liquid water is taken as unity in calculations of the equilibrium constant.

The standard free energy of formation, $\Delta F_f°$, for hydrogen ion (pH 0) is zero by definition. The free energy of formation, $\Delta F_f°'$, for hydrogen ion at pH 7 is taken as —9.93 kcal. at 310°K and —9.52 kcal. at 298°K.

Values for free energies of formation are given per mole in aqueous solution. The free energy change associated with a given reaction is for the number of moles prefixed to reactants and products in the chemical equation.

It is to be noted that the $\Delta F_f°$ of a given ionic species (except H^+) at pH 0 can be used in calculation of the free energy change of a reaction in which such species appears, regardless of pH. A value of $\Delta F_f°$ for a given ionic species may be calculated even though this may not be the predominant species at the reference pH, namely zero. It is unnecessary to specify how a 1 molar solution of such an ion at pH 0 can be obtained. In other words, except for hydrogen ion, $\Delta F_f°$ can be considered to be equal to $\Delta F_f°'$.

Changes in the free energy have been calculated from either

$$\Delta F° = \Sigma\Delta F_f° \text{ products} - \Sigma\Delta F_f° \text{ reactants} \tag{1}$$

or

$$\Delta F_{310}° = -RT\ln K_{eq} = -1.419 \log_{10} K_{eq} = 1.419(pK_{eq}) \text{ kcal.} \tag{2}$$

Many of the values for standard free energy changes found in the literature have been determined from the well known thermodynamic relationship:

$$\Delta F° = \Delta H° - T\Delta S° \tag{3}$$

III. Structure and Properties of Ammonia

The ammonia molecule is a flat pyramid in which the nitrogen atom is situated above the plane formed by 3 hydrogen atoms. Passage of the nitrogen atom from one equilibrium position above the plane to the other equilibrium position below the plane has associated with it an "inversion spectrum" in the microwave region (*6*). The molecule is extremely soluble in water, approximately 700 volumes dissolving in one volume of water at 20° to form a solution of ammonium hydroxide.

Aside from its well known basic properties, liquid ammonia, like water, shows weak acid properties, dissociating into H^+ and NH_2^- ions ($K = 10^{-16}$ at $-33°$). Some important properties and values (7) appear in Table I.

TABLE I

PROPERTIES OF AMMONIA[a,b]

Melting point	195.36°K;	ΔH (fusion): 1.352 kcal. × mole^{-1}
Boiling point	239.68°K;	ΔH (vaporization): 5.581 kcal. × mole^{-1}
Height of molecule	0.360 Å	
Bond distance	1.016 Å	
Distance, H—H	1.645 Å	
Dipole moment	1.466×10^{-18} esu	
Entropy, $S_{298}°$	NH_3 (gas),	45.94 cal. × deg^{-1} × mole^{-1}
	NH_4^+ (aq.)	26.4 cal. × deg^{-1} × mole^{-1}
$\Delta F°$ (formation)	NH_3 (gas)	-3.940 kcal. × mole^{-1}
	NH_3 (aq.)	-6.330 kcal. × mole^{-1}
	NH_4OH (aq.)	-62.99 kcal. × mole^{-1}
	NH_4^+ (aq.)	-18.96 kcal. × mole^{-1}

[a] Adapted from Yost and Russell (7); Latimer (4).

[b] $NH_4OH = NH_4^+ + OH^-$: $K_{298} = 1.81 \times 10^{-5}$; $\Delta F_{298}° = 6.47$ kcal. × mole^{-1}.

A special feature of the ammonia molecule may be mentioned. Ammonia and the free amino (un-ionized) and amide groups of amino acids and proteins possess an unshared pair of electrons. Such groups can complex with various metals to form coordination compounds (complexes). These unshared electrons are of biological importance. They contribute to the fact that amino acids possess a pK_a in the basic region at about pH 10. Charged amino groups contribute to the stabilization of specific protein structures.

The only isotope of nitrogen suitable for biological tracer work is the stable isotope N^{15} which has found extensive application. Deuteroammonia is unsuitable for tracer work because equilibrium with hydrogen ions is rapidly attained in water solution.

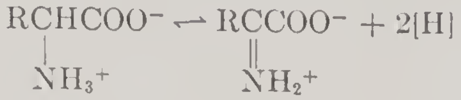
IV. Formation of Ammonia

A. OXIDATIVE DEAMINATION

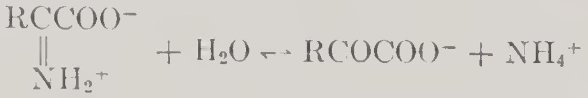
1. *Enzymes Catalyzing the Oxidative Deamination of Amino Acids*

For purposes of simplicity, the enzymes catalyzing the oxidative deamination of amino acids will be considered under the following classification: (*a*) L-amino acid oxidases, (*b*) D-amino acid oxidases, and (*c*) specific amino acid oxidases. Several comprehensive reviews of this general topic have appeared in recent years (*8–10*).

The general reaction for the oxidative deamination of an amino acid may be formulated as follows:

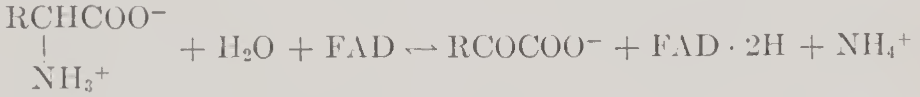


$$\underset{\substack{|\\ NH_3^+}}{RCHCOO^-} \rightleftharpoons \underset{\substack{\|\\ NH_2^+}}{RCCOO^-} + 2[H] \qquad (4)$$



$$\underset{\substack{\|\\ NH_2^+}}{RCCOO^-} + H_2O \rightleftharpoons RCOCOO^- + NH_4^+ \qquad (5)$$

Reaction 5 is nonenzymatic. It should be noted that the primary reaction is that of a dehydrogenation and thus the term dehydrogenase is appropriate for the enzyme. However, because of precedent and the obligatory role of oxygen as hydrogen acceptor in certain systems, the term oxidase continues to be used. The above classification indicates that the L- and D-amino acid oxidases have broad substrate specificities in contrast to the specific amino acid oxidases. The D- and L-amino acid oxidases as far as they have been studied are flavoproteins, and the reaction in these cases may be represented as follows:



$$\underset{\substack{|\\ NH_3^+}}{RCHCOO^-} + H_2O + FAD \rightleftharpoons RCOCOO^- + FAD \cdot 2H + NH_4^+ \qquad (6)$$

$$FAD \cdot 2H + O_2 \rightarrow FAD + H_2O_2 \qquad (7)$$

$$\Delta F^{\circ\prime} \text{ (over-all)} \cong -20 \text{ kcal.}$$

a. L-*Amino Acid Oxidases.* Systematic studies have been carried out on L-amino acid oxidases from snake venoms and tissues (*11*), rat kidney (*12*), turkey (*Meleagris gallopavo* L.) liver (*13*), digestive gland of the mollusk *Mytilus edulis* (*14*), *Neurospora crassa* (*15–17*), *Penicillium* (*18*), *Aspergillus niger* (*18*), and *Proteus vulgaris* (*19*). The L-amino acid oxidases from snake venom (*20*), *Neurospora crassa* (*17*) and turkey liver (*13*) have been highly purified. The substrate specificity of the different enzymes varies with respect to individual amino acids (*9, 10, 15*) but all are capable of catalyzing the oxidative deamination of a large number of L-amino acids. The L-amino acid oxidase from rat kidney (*12*) is the least active of these enzymes.

b. D-*Amino Acid Oxidases.* D-Amino acids have a relatively limited distribution except in the case of certain microorganisms (*10*). The relatively widespread occurrence and high activity of D-amino acid oxidase thus represents an enigma in that the functional role of this enzyme remains unexplained. In the mammal, D-amino acid oxidase is found only in liver and kidney. D-Amino acid oxidases have been found in the livers of cephalopods, gastropods, and the lamellibranch *Mytilus edulis* (but not *Anodonta*) (*21*), and have been shown to be present in the following microorganisms: *Neurospora crassa* (*15, 16*), *Aspergillus niger* (*22*), *Penicillium chrysogenum* (*22*), *P. notatum* (*22*), and *P.*

roqueforti (*22*), *Proteus vulgaris* (*23*), and *Pseudomonas aeruginosa* (*24*). The substrate specificity is broad for the different D-amino acid oxidases but activity varies with respect to individual amino acids. D-Amino acid oxidase is reported (*24a*) to occur in the fat body and Malpighian tubes of the American and German cockroaches, and in the fat body of the greater wax moth, *Galleria mellonella*.

c. Specific Amino Acid Oxidases. The enzymes to be considered in this category are those which catalyze the oxidation of a single (or a few closely related) amino acids.

(*1*) D-*Glutamic acid oxidase.* Cephalopod liver is reported to contain a relatively specific D-glutamic acid oxidase (*25*) in addition to a D-amino acid oxidase of broad specificity.

Mycelia of *Aspergillus ustus*, strain *f*, have been reported to contain a D-amino acid oxidase specific for D-glutamic and D-aspartic acids (*26*). The enzyme is twice as active toward the former as the latter. Other D-amino acids are not oxidized by this enzyme. Studies with purified enzyme preparations confirmed the specificity toward glutamic and aspartic acids (*27*).

(*2*). D-*Aspartic acid oxidase.* Rabbit kidney and liver have been reported to contain an enzyme specific for D-aspartic acid (*28, 29*). Rat liver does not contain this enzyme (*30*).

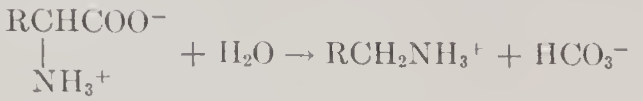
(*3*). L-*Alanine dehydrogenase.* A mutant form of *Bacillus subtilis* (S–) has been reported to contain a highly active and specific enzyme for L-alanine (*31*). The enzyme has been shown to require DPN as coenzyme [see also Goldman (*31a*).]

(*4*). *Glycine oxidase.* Kidney and liver of mammals [dog, human, lamb, ox, pig, rabbit, and rat (liver only)] contain an enzyme which catalyzes the oxidation of glycine (*32*). FAD is required as coenzyme.

(*5*). *Glutamic acid dehydrogenase.* Glutamic acid dehydrogenase is practically ubiquitous in plants, microorganisms, and animals. Aside from its wide distribution, it is highly active and is the only enzyme concerned with oxidative deamination utilizing DPN or TPN. [An exception to this is seen in the rare instance, previously referred to (*31*), of an L-alanine dehydrogenase existing as a DPN enzyme in a mutant form of *B. subtilis*.] The metabolic significance of glutamic acid dehydrogenase will be referred to in later discussion.

2. *Enzymes Catalyzing the Oxidation of Amines*

The occurrence of mono-, di-, and polyamines in plants, microorganisms, and animal tissues is well known. The simple mono- and diamines are presumably formed as a result of decarboxylation of the corresponding amino acids by specific amino acid decarboxylases [see review by Schales (*33*)], which require pyridoxal phosphate as coenzyme.



$$\underset{\displaystyle NH_3^+}{\overset{\displaystyle RCHCOO^-}{|}} + H_2O \rightarrow RCH_2NH_3^+ + HCO_3^- \qquad (8)$$

$$\Delta F^{\circ\prime} \cong -5 \text{ kcal.}$$

The enzymes catalyzing the oxidative deamination of amines are widely distributed (*34, 35*) and have been classified under two categories, monoamine oxidase and diamine oxidase, suggesting that those catalyzing the oxidation of monoamines are different from those concerned with the oxidation of diamines. While considerable evidence has accumulated supporting this, studies by Mann (*36*) with a highly purified amine oxidase from pea seedlings indicate that this enzyme catalyzes the oxidative deamination of mono- as well as diamines. Fouts *et al.* (*37*) found that a partially purified hog kidney preparation of diamine oxidase was able to catalyze the oxidation of 13 different monoamines. Mann (*36*) has presented evidence that the enzyme from peas is a metalloprotein. Flavin mononucleotide (*38, 39*) and pyridoxal phosphate (*40*) have been proposed as coenzymes for amine oxidase preparations. Goryachenkova (*41*) has reported that both are required. The oxidation of amines can be formulated as shown (Reactions 9–10b).

$$RCH_2NH_3^+ + FMN \rightleftharpoons RCH{=}NH_2^+ + FMN \cdot 2H \qquad (9)$$

$$FMN \cdot 2H + O_2 \rightarrow FMN + H_2O_2 \qquad (10)$$

$$RCH{=}NH_2^+ + H_2O \rightarrow RCHO + NH_4^+ \qquad (10a)$$

$$\text{Net:} \quad RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_4^+ \qquad (10b)$$

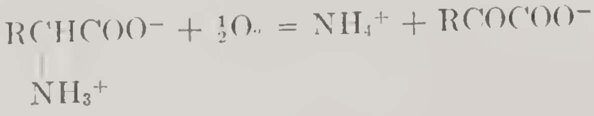
Histaminase is apparently identical with diamine oxidase. It is rather peculiar that the straight-chain diamines of 14–18 carbon atoms are oxidized by monoamine oxidase but not by diamine oxidase. The substrate specificity and activity vary considerably with the source of the enzyme.

Amine oxidases appear to be widely distributed in plants (*42, 43*) and in tissues of all vertebrates and in many invertebrates (*35, 44–47*).

In all animals studied, liver, kidney (except the rat) and intestine show the highest activity. Placenta has been reported to have high activity (*48, 49*). Human plasma contains an amine oxidase differing in substrate specificity from the usual mono- and diamine oxidases (*50*). Amine oxidases are present in *Pseudomonas pyocyanea* (*51, 52*), *Mycobacterium smegmatis* (*53, 54*), and *Escherichia coli* (*54, 55*).

3. *Energetics of Ammonia Formation*

The general reaction,



$$\underset{\displaystyle NH_3^+}{\overset{\displaystyle RCHCOO^-}{|}} + \tfrac{1}{2}O_2 = NH_4^+ + RCOCOO^- \qquad (11)$$

has associated with it a free energy change, $\Delta F^{\circ\prime}_{298}$, of the order of —44 kcal. If H_2O_2 is formed as in Reactions 6 and 7, the standard free energy change is of the order of —20 kcal.

In general the formation of ammonia *per se* by oxidative deamination or other mechanisms leads to no net gain of useful energy, other than heat, to the cell since there are no coupling mechanisms available for synthesis of ATP at this step. In the case of the amino acids the potential energy made available in the course of oxidative deamination would be made available ultimately as ATP if a common pathway involving a pyridine nucleotide as the initial hydrogen or electron acceptor were utilized.

In view of the fact that most cells lack an L-amino acid oxidase of sufficient activity to account for the oxidative deamination of the different amino acids, it has been proposed, initially by Braunstein and Asarkh (*56*, *56a*, *57*) that oxidative deamination occurs as a result of coupling transaminase systems and the glutamic acid dehydrogenase system, as shown in Fig. 2. In support of their proposal are the following: (*a*) transaminases have been shown to be present in cells and tissues capable of converting α-ketoglutaric acid to glutamic acid in the presence of most L-amino acids (*9*, *10*, *56a*); (*b*) glutamic acid dehydrogenase has a ubiquitous distribution and requires DPN or TPN.

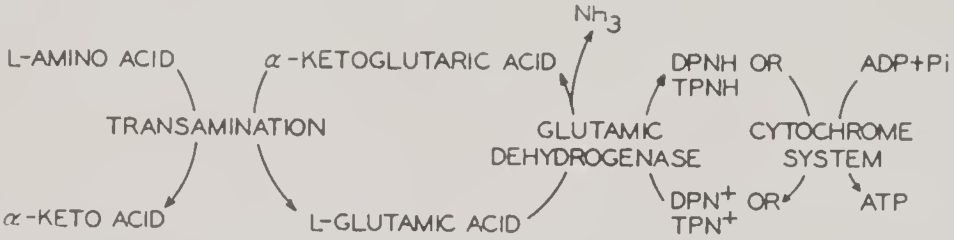


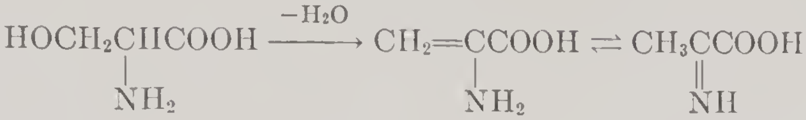
FIG. 2. Scheme for deamination of amino acids via coupled transaminase(s) and glutamic dehydrogenase.

The net effect of this scheme from an energetic standpoint would be that of generating ATP by utilizing the energy of oxidation of the reduced pyridine nucleotides formed during the oxidation of glutamic acid. Deamination of amino acids, amines or other substrates not linked with the pyridine nucleotides would result in liberation of the energy of deamination in the form of heat. For a more detailed and comprehensive discussion of this topic, the reader is referred to the recent review by Krebs and Kornberg (*1*).

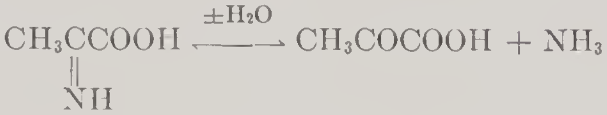
B. NONOXIDATIVE DEAMINATION OF AMINO ACIDS

1. *Amino Acid Dehydrases*

The hydroxy amino acids, serine, threonine, and homoserine, undergo deamination in the presence of specific enzymes which appear to catalyze a primary dehydration followed by a spontaneous deamination. In the case of serine, the reactions (12, 13) may be formulated as shown.



$$\mathrm{HOCH_2\underset{\underset{\displaystyle NH_2}{|}}{C}HCOOH \xrightarrow{-H_2O} CH_2{=}\underset{\underset{\displaystyle NH_2}{|}}{C}COOH \rightleftharpoons CH_3\underset{\underset{\displaystyle NH}{\|}}{C}COOH} \qquad (12)$$



$$\mathrm{CH_3\underset{\underset{\displaystyle NH}{\|}}{C}COOH \xrightleftharpoons{\pm H_2O} CH_3COCOOH + NH_3} \qquad (13)$$

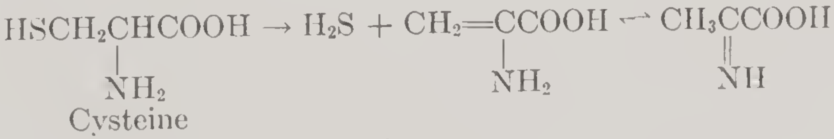
Threonine and homoserine follow similar pathways with the resulting formation of α-ketobutyric acid and NH_3. For the reaction

$$\text{Threonine}^{\pm} = \alpha\text{-Ketobutyrate}^{-} + \mathrm{NH_4^+}, \qquad (13a)$$
$$\Delta F^{\circ\prime} \cong -7 \text{ kcal.}$$

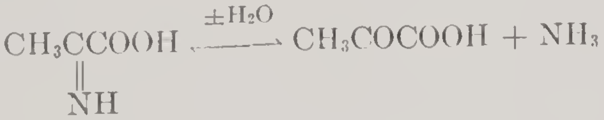
Pyridoxal phosphate is the coenzyme for these dehydrases. Amino acid dehydrases active with both D and L forms of the amino acids have been shown to be present in *Escherichia coli* (*57a–61*), *Pseudomonas pyocyanea* (*57a*), *Proteus* OX-19 (*57a*), *Clostridium welchii* (*57a*), and *Neurospora crassa* (*62, 63*). Dehydrases active for the L forms of serine, threonine, and homoserine are present in mammalian liver (*57a, 64, 65*). Serine and threonine dehydrases have been highly purified from sheep liver (*66*).

2. *Amino Acid Desulfhydrases*

The sulfur-containing amino acids cysteine and homocysteine undergo catalytic desulfhydration with the subsequent formation of ammonia. The reactions (14, 15) may be formulated as shown.



$$\mathrm{\underset{Cysteine}{HSCH_2\underset{\underset{\displaystyle NH_2}{|}}{C}HCOOH} \rightarrow H_2S + CH_2{=}\underset{\underset{\displaystyle NH_2}{|}}{C}COOH \rightleftharpoons CH_3\underset{\underset{\displaystyle NH}{\|}}{C}COOH} \qquad (14)$$

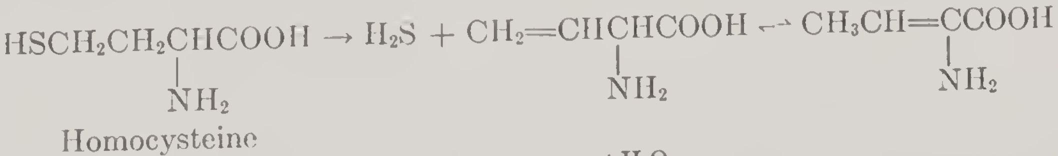


$$\mathrm{CH_3\underset{\underset{\displaystyle NH}{\|}}{C}COOH \xrightleftharpoons{\pm H_2O} CH_3COCOOH + NH_3} \qquad (15)$$

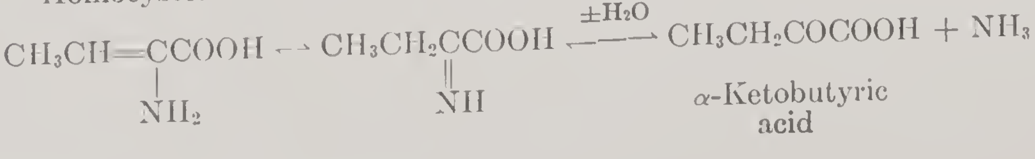
For the over-all reaction,

$$\text{Cysteine}^{\pm} + \mathrm{H_2O} = \text{Pyruvate}^{-} + \mathrm{HS^-} + \mathrm{NH_4^+} + \mathrm{H^+} \qquad (15a)$$
$$\Delta F^{\circ\prime} = -7 \text{ kcal.}$$

The analogous reactions (16, 17) for homocysteine may be formulated as shown.



$$\mathrm{\underset{Homocysteine}{HSCH_2CH_2\underset{\underset{\displaystyle NH_2}{|}}{C}HCOOH} \rightarrow H_2S + CH_2{=}CH\underset{\underset{\displaystyle NH_2}{|}}{C}HCOOH \rightleftharpoons CH_3CH{=}\underset{\underset{\displaystyle NH_2}{|}}{C}COOH} \qquad (16)$$

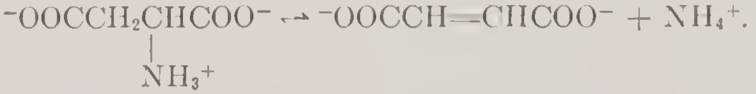


$$\mathrm{CH_3CH{=}\underset{\underset{\displaystyle NH_2}{|}}{C}COOH \rightleftharpoons CH_3CH_2\underset{\underset{\displaystyle NH}{\|}}{C}COOH \xrightleftharpoons{\pm H_2O} \underset{\alpha\text{-Ketobutyric acid}}{CH_3CH_2COCOOH} + NH_3} \qquad (17)$$

Desulfhydrases have been reported to be present in microorganisms and animal tissues (*67, 68*) (liver, kidney, and pancreas). *E. coli* is active with D-cysteine (*69*). The enzymes require pyridoxal phosphate as coenzyme (*70, 71*). Kallio (*70*) has partially purified the cysteine and homocysteine desulfhydrases from *P. morganii.*

3. *Aspartase*

Aspartase catalyzes the following reaction:



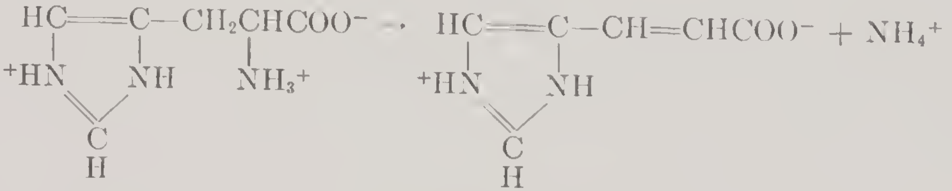
$$^-OOCCH_2\underset{\substack{|\\ NH_3^+}}{C}HCOO^- \rightleftharpoons {}^-OOCCH{=}CHCOO^- + NH_4^+. \qquad (18)$$

$$\Delta F^{\circ\prime} = 2.7 \text{ kcal.}$$

The enzyme has been found to occur in microorganisms and plant tissues but not in animal tissues (*72*). A highly purified aspartase preparation has been made from *Bacterium cadaveris* (*73*). A comparison of K_m values for the purified enzyme from *B. cadaveris* with other enzyme preparations from *E. coli, Proteus X19, Ps. fluorescens* and *Propionibacterium peterssonii* is recorded by these investigators. The purified system appeared to require no cofactors. The role of aspartase in ammonia fixation is discussed in a later section.

4. *Histidase*

Histidase catalyzes Reaction 19.



(19)

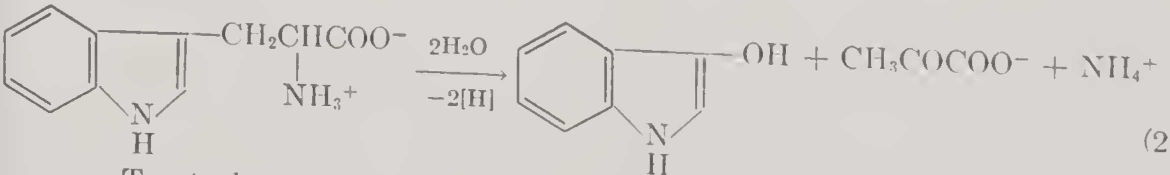
Histidine Urocanic acid

This enzyme has been the subject of two recent reviews (*74, 75*). The free energy change associated with Reaction 19 is possibly about 3 kcal.

Histidase has been found in the liver of all animals studied and is also present in bacteria. As far as is known there are no specific cofactors required.

5. *Tryptophanase*

E. coli contains an enzyme(s) which catalyzes the over-all Reaction 20.



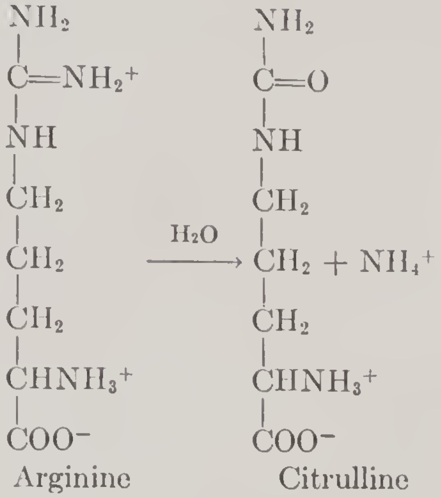
(20)

Tryptophan Indole Pyruvic acid

Pyridoxal phosphate is a coenzyme for this system (*76, 77*).

6. *Arginine Desiminase*

Microorganisms contain an enzyme which catalyzes Reaction 21.

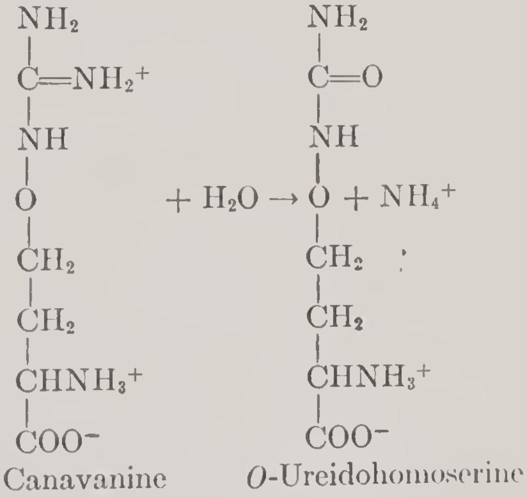


$$\begin{array}{l} \mathrm{NH_2} \\ | \\ \mathrm{C{=}NH_2^+} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CHNH_3^+} \\ | \\ \mathrm{COO^-} \end{array} \xrightarrow{\mathrm{H_2O}} \begin{array}{l} \mathrm{NH_2} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CHNH_3^+} \\ | \\ \mathrm{COO^-} \end{array} + \mathrm{NH_4^+} \qquad (21)$$

Arginine Citrulline

$\Delta F^\circ \simeq -6$ kcal.

Some aspects of this enzyme system have been recently reviewed (*78–80*). The enzyme has been shown to be present in *Streptococcus faecalis, Pseudomonas aeruginosa, Clostridium perfringens,* and bakers' yeast. The enzyme from *S. faecalis* has been partially purified and shown to have no cofactor requirements (*81*). *Streptococcus faecalis* extracts were found to be capable of catalyzing Reaction 22 (*82*).



$$\begin{array}{l} \mathrm{NH_2} \\ | \\ \mathrm{C{=}NH_2^+} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{O} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CHNH_3^+} \\ | \\ \mathrm{COO^-} \end{array} + \mathrm{H_2O} \rightarrow \begin{array}{l} \mathrm{NH_2} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{O} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CHNH_3^+} \\ | \\ \mathrm{COO^-} \end{array} + \mathrm{NH_4^+} \qquad (22)$$

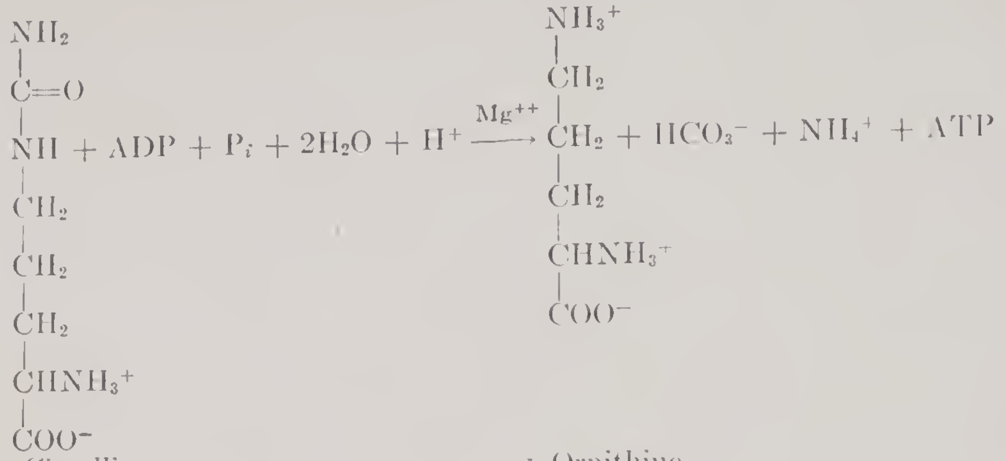
Canavanine *O*-Ureidohomoserine

$\Delta F^\circ \simeq -6$ kcal.

The enzyme involved is apparently the same as that acting on arginine to form citrulline (arginine desiminase).

7. *Citrulline Phosphorylase*

An enzyme system capable of forming ammonia from citrulline is present in certain microorganisms [*Streptococcus faecalis* (*83–86*), *Streptococcus lactis* (*87, 88*), *Pseudomonas,* strain P5 (*89*)]. The reaction (23) may be represented as shown.



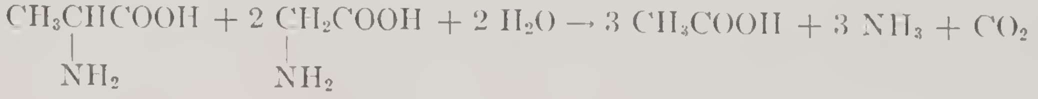
$$NH_2\text{–}C(=O)\text{–}NH\text{–}CH_2\text{–}CH_2\text{–}CH_2\text{–}CHNH_3^+\text{–}COO^- + ADP + P_i + 2H_2O + H^+ \xrightarrow{Mg^{++}} NH_3^+\text{–}CH_2\text{–}CH_2\text{–}CH_2\text{–}CHNH_3^+\text{–}COO^- + HCO_3^- + NH_4^+ + ATP \quad (23)$$

L-Citrulline L-Ornithine

The enzyme components of this system are discussed in Section VII. These enzymes are concerned in citrulline biosynthesis and breakdown in *S. faecalis* (*90, 91*) and in citrulline biosynthesis in mammalian liver (*92–95*). While the breakdown of citrulline is of great importance energetically in microorganisms, it is of questionable significance in animal metabolism. These enzymes are also concerned with the biosynthesis of arginine and urea (Section VII).

8. *Miscellaneous Nonoxidative Amino Acid Deaminases*

Anaerobic microorganisms, particularly the Clostridiae, are able to produce NH_3 from amino acids by means of a coupled deamination reaction in which one amino acid acts as the donor and the other as the acceptor of hydrogen. This type of reaction system has been referred to as the Stickland reaction (*96–101*). Reactions 24 and 25 represent type reactions.



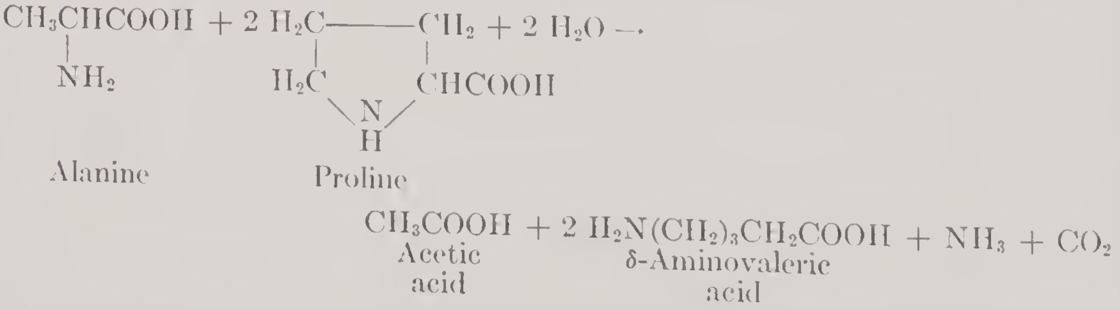
$$CH_3CH(NH_2)COOH + 2\ CH_2(NH_2)COOH + 2\ H_2O \rightarrow 3\ CH_3COOH + 3\ NH_3 + CO_2 \quad (24)$$

Alanine Glycine Acetic acid

For the reaction

$$\text{Alanine}^{\pm} + 2\ \text{Glycine}^{\pm} + 3\ H_2O = 3\ \text{Acetate}^- + 3\ NH_4^+ + HCO_3^- + H^+ \quad (24a)$$

$$\Delta F_{298}{}^{\circ\prime} = -36.5\ \text{kcal.}$$

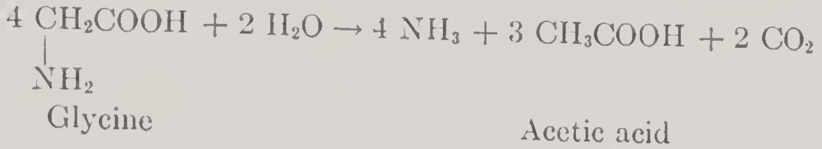


$$CH_3CH(NH_2)COOH + 2\ \text{Proline} + 2\ H_2O \rightarrow CH_3COOH + 2\ H_2N(CH_2)_3CH_2COOH + NH_3 + CO_2 \quad (25)$$

Alanine Proline

Acetic acid δ-Aminovaleric acid

A somewhat different type of fermentation is carried out by the organism *Diplococcus glycinophilus* (*102*) and may be formulated as shown (Reaction 26).



$$4\,\underset{\text{Glycine}}{\underset{\underset{NH_2}{|}}{CH_2}COOH} + 2\,H_2O \rightarrow 4\,NH_3 + 3\,\underset{\text{Acetic acid}}{CH_3COOH} + 2\,CO_2 \qquad (26)$$

For the reaction

$$4\ \text{Glycine}^{\pm} + 4\,H_2O = 4\,NH_4^+ + 3\ \text{Acetate}^- + 2\,HCO_3^- + H^+ \qquad (26a)$$
$$\Delta F_{298}^{\circ\prime} = -49\ \text{kcal.}$$

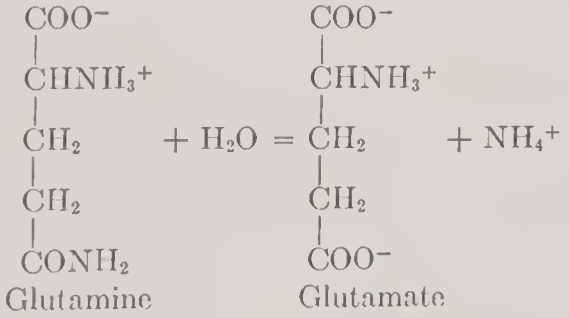
It is beyond the scope of this chapter to review the great variety of amino acid fermentation reactions carried out by microorganisms. The subject has been reviewed by Nisman (*103*).

C. Amidases

A number of amides are known to occur naturally, the best known being glutamine, asparagine and urea. Enzyme systems capable of hydrolyzing these amides have been studied in plant tissues, microorganisms, and animal tissues.

1. *Glutaminase*

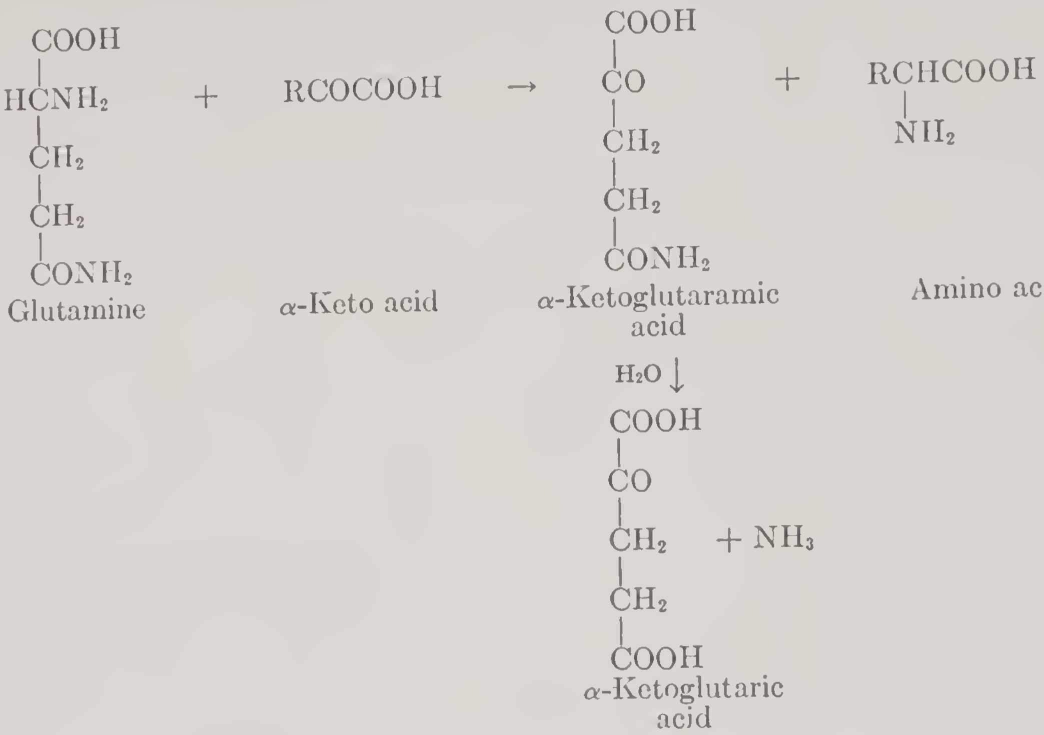
Glutamine is almost ubiquitous in living cells and has been assigned special metabolic roles in plants, microorganisms and animals. Glutamine exists in proteins in peptide linkage. Comprehensive reviews of glutaminases and glutamine metabolism have appeared in recent years (*10, 104–108*). Deamidation of glutamine in animal tissues may occur as the result of two types of enzyme catalyzed reactions. Glutaminase I catalyzes Reaction 27 and is activated by anions such as phosphate (*109, 110*).



$$\begin{array}{lcll} COO^- & & COO^- & \\ | & & | & \\ CHNH_3^+ & & CHNH_3^+ & \\ | & & | & \\ CH_2 & + H_2O = & CH_2 & + NH_4^+ \\ | & & | & \\ CH_2 & & CH_2 & \\ | & & | & \\ CONH_2 & & COO^- & \\ \text{Glutamine} & & \text{Glutamate} & \end{array} \qquad (27)$$

$$\Delta F_{310}^{\circ\prime} = -3.5\ \text{kcal.}$$

The second type of glutamine deamidation (Glutaminase II) requires the participation of an α-keto acid (*111, 112*) and has been shown to involve a primary transamination (*113*) (Reactions 28 and 29).



$$\underset{\text{Glutamine}}{\mathrm{HOOC{-}CH(NH_2){-}CH_2{-}CH_2{-}CONH_2}} + \underset{\alpha\text{-Keto acid}}{\mathrm{RCOCOOH}} \rightarrow \underset{\alpha\text{-Ketoglutaramic acid}}{\mathrm{HOOC{-}CO{-}CH_2{-}CH_2{-}CONH_2}} + \underset{\text{Amino acid}}{\mathrm{RCH(NH_2)COOH}} \quad (28)$$

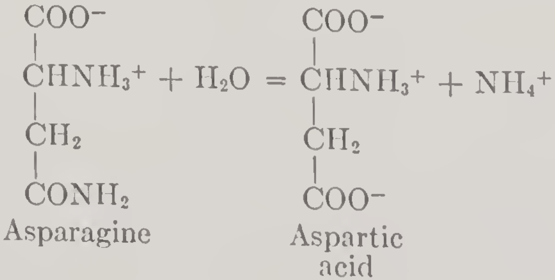
$$\mathrm{HOOC{-}CO{-}CH_2{-}CH_2{-}CONH_2} \xrightarrow{H_2O} \underset{\alpha\text{-Ketoglutaric acid}}{\mathrm{HOOC{-}CO{-}CH_2{-}CH_2{-}COOH}} + \mathrm{NH_3} \quad (29)$$

This enzyme is essentially limited to liver in its distribution although kidney may have slight activity (*114*). Glutaminase I activity of animal tissues has been systematically studied by Krebs (*115*) and by Greenstein and associates (*109–112*), and in microorganisms by McIlwain (*116*). The enzyme has been partially purified from *Clostridium welchii* (*117*), *E. coli* (*118*), rat liver (*119, 120*), and hog kidney (*121*).

Glutaminase activity in the animal kidney has been correlated with urinary ammonia production (*122–125*). On the other hand, glutaminase activity does not appear to account for ammonia production in brain (*126*). Glutaminase activity has been demonstrated in tissues of the fetal pig (46 days gestation) (*126a*); activities, corrected for endogenous ammonia production, were observed to be in the following order: chorioallantoic membrane > metanephros > amniotic membrane > allantoic membrane > mesonephros.

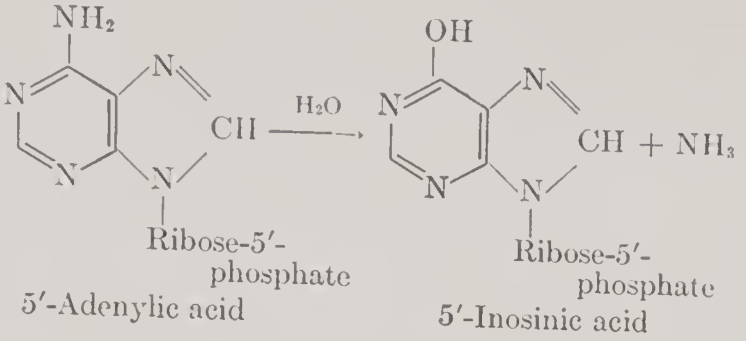
2. *Asparaginase*

Asparaginase, while widely distributed in microorganisms, plants, and animal tissues (*105*), has not been studied as extensively as glutaminase chiefly because of its relatively low activity in most cells.



$$\underset{\text{Asparagine}}{\mathrm{{}^-OOC{-}CHNH_3^+{-}CH_2{-}CONH_2}} + \mathrm{H_2O} = \underset{\text{Aspartic acid}}{\mathrm{{}^-OOC{-}CHNH_3^+{-}CH_2{-}COO^-}} + \mathrm{NH_4^+} \quad (30)$$

$\Delta F^{\circ\prime} = -3.5$ kcal.

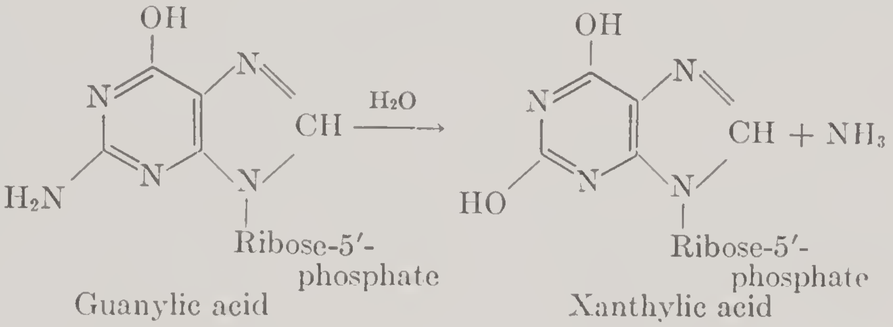


(34)

also present in other tissues (*141, 142*). The enzyme has been studied in blood of different animals (*141, 142*), in rat and pigeon muscle (*143*), and in brain (*144*). Rabbit skeletal muscle was found to have about 25 times greater activity than the claw muscle of the crab (*142*). Substrate specificity studies with the crystalline enzyme indicate that it is specific for 5′-adenylic acid (*140*).

b. 3′-Adenylic Acid Deaminase. Rabbit liver extracts have been reported to catalyze the deamination of yeast adenylic acid (3′-adenylic acid) (*145*).

c. Guanylic Acid Deaminase. This enzyme is present in mammalian liver (*138, 146*) and catalyzes the reaction shown (35).



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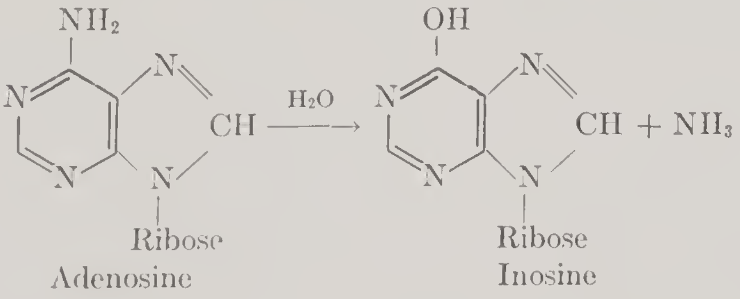
2. *Nucleoside Deaminases*

a. Adenosine Deaminase. This enzyme, which catalyzes Reaction 36,



(36)

was first shown to be present in rabbit muscle (*139*) and has since been found in most tissues of the rabbit (*142*). Highest activity occurs in the intestinal tract (*142*). Partial purification from calf intestinal mucosa has been reported (*147, 148*).

The enzyme from calf intestine deaminates deoxyadenosine at approximately the same rate as adenosine. In contrast, rabbit blood, plasma, ventricle, auricle, jejunum, duodenum, and thymus preparations deaminate deoxyadenosine at only one-half the rate of adenosine. Whole blood of chicken, rat, and the human also were found to be more active in deaminating adenosine (*147*).

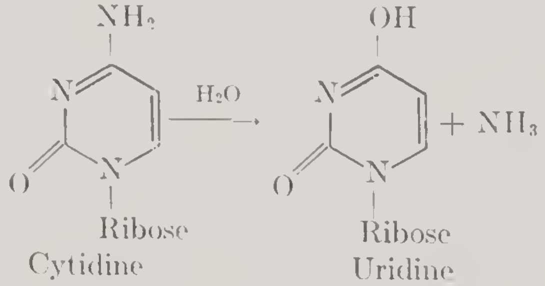
Roush and Betz (*137*) recently reported the presence of adenosine deaminase in lobster hepatopancreas. These workers found no adenase in this organ, an observation in contrast to the report by Duchateau-Bosson *et al.* (*149*).

Mitchell and McElroy (*150*) partially purified adenosine deaminase from *Aspergillus oryzae*. Further studies with purified preparations from this organism by Kaplan *et al.* (*151*) revealed that the enzyme deaminated not only adenosine, but also 5′– and 3′–adenylic acid, DPN, ATP, ADP, and ADPR (adenosine diphosphate-ribose). 5′-Adenylic acid was found to be deaminated at one-half the rate of adenosine. Adenine, TPN, and 2′-adenylic acid were not deaminated.

Lactobacillus helveticus extracts were found to deaminate adenosine but not deoxyadenosine. This is in contrast to the specificity of the enzymes from mammalian tissues as well as from *E. coli* and *Lb. casei* which are able to deaminate both (*152*).

Disintegrated resting spore preparations of *Bacillus cereus* were found by Powell and Hunter (*153*) to deaminate adenosine and cytidine but not adenine, guanosine, guanine, or cytosine. The possible relationship of this enzyme to the stimulating activity of inosine on spore germination is discussed by these workers.

b. Cytidine Deaminase. This enzyme converts cytidine to uridine (Reaction 37).

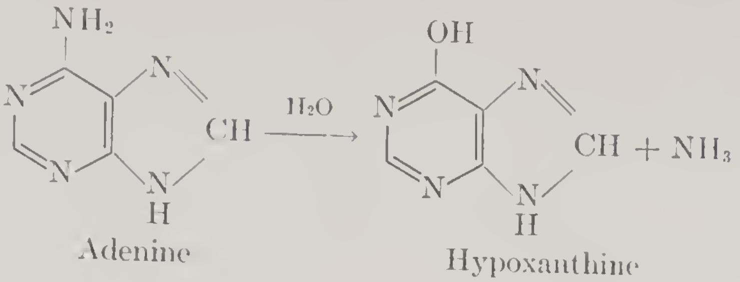


(37)

Extracts of *E. coli* deaminate cytidine and deoxycytidine but not adenine, adenosine, cytosine, isocytosine, guanine, or guanosine (*154, 155*).

3. *Aminopurine and Aminopyrimidine Deaminases*

a. Adenase (Adenine Deaminase). This enzyme converts adenine to hypoxanthine (Reaction 38).



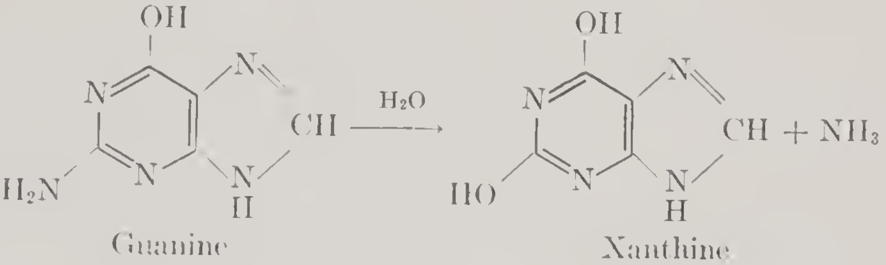
(38)

For the reaction

$$\text{Adenine}(solid) + H_2O = \text{Hypoxanthine}(solid) + NH_3(aqueous) \quad (38a)$$
$$\Delta F_{298}° = -2.8 \text{ kcal.}$$

As previously mentioned, adenase is not widespread in nature. Greenstein (*138*) and Wakabayasi (*145*) could find no evidence for the presence of this enzyme in a variety of animal tissues. The enzyme has been reported to be present in cow's milk (*156*), in rat blood (*157*), and in crayfish hepatopancreas (*149*). However, lobster hepatopancreas does not contain this enzyme (*137*). *Torulopsis utilis* contains adenase which can be increased in amount by growing the yeast in the presence of adenine (*158*).

b. Guanase (Guanine Deaminase). Guanine is deaminated to xanthine as shown (Reaction 39).



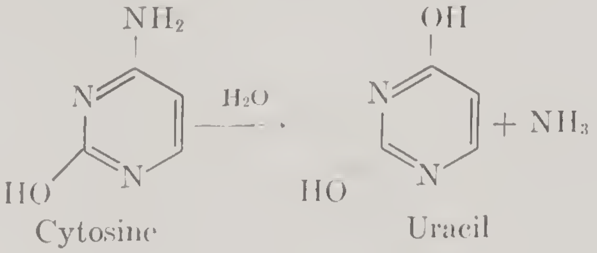
(39)

For the reaction

$$\text{Guanine}(solid) + H_2O = \text{Xanthine}(solid) + NH_3(aqueous) \quad (39a)$$
$$\Delta F_{298}° = -0.6 \text{ kcal.}$$

The separation of this enzyme from deaminases for guanylic acid and guanosine was achieved by Schmidt and by Wakabayasi (*145*). Rat skin extracts have guanase but no adenase while human skin lacks both enzymes (*159*).

c. Cytosine Deaminase. Extracts of *E. coli* and yeast were found to contain cytosine deaminase (*160*).

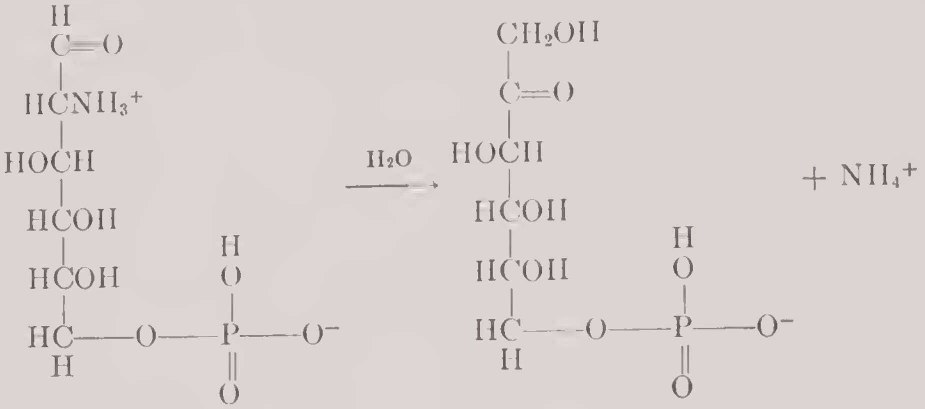


(40)

E. Hexosamine Deaminases

1. *Glucosamine-6-phosphate Deaminase*

Glucosamine-6-phosphate is deaminated by *E. coli* extracts to yield ammonia and fructose-6-phosphate (*161, 162*). A similar enzyme is present in liver and kidney (*163*).



(41)

Glucosamine-6-phosphate
Fructose-6-phosphate

V. Inorganic Nitrogen Metabolism*

A. Nitrification

Oxidation of ammonium ions or other nitrogenous compounds to nitrite or nitrate by autotrophic soil organisms is termed nitrification. Aerobic chemosynthetic autotrophes, members of the family Nitrobacteriacae, derive energy from such oxidations (*165–167*). Some heterotrophic microorganisms may also carry out nitrification.

Nitrosomonas, first isolated in pure culture by Winogradsky in 1891, oxidizes ammonia to nitrite in the 6-electron redox reaction formulated by Godlewski and by Meyerhoff [cf. Delwiche (*165*)] as

$$NH_4^+ + \tfrac{3}{2}\,O_2 = 2\,H^+ + H_2O + NO_2^- \qquad (42)$$
$$\Delta F_{298}{}^\circ = -46.2 \text{ kcal.}$$

The standard free energies of formation of NH_4^+ (−19.0 kcal.), NO_2^- (−8.45 kcal.), and H_2O (−56.7 kcal.) yield −46.2 kcal. for the standard free energy change in the above exergonic reaction. The free energy change at physiological concentrations is likely to be much higher (*168*), because of the formation of hydrogen ions at pH 8. For example, with NO_2^- and NH_4^+ at equal concentrations at pH 8, and with an oxygen partial pressure of 0.2 atm., $\Delta F^\circ_{298} = -67.0$ kcal. The oxidation of ammonia to nitrite could conceivably take place in three

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[*] For a recent treatment of this subject and for keys to the older literature see McElroy and Glass (*164*). See also Nason and Takahashi (*164a*).

2-electron steps (*169*). Possible intermediates in such steps are discussed in a recent symposium (*164*).

Information as to energy-coupling reactions is lacking, and until intermediate steps in the over-all oxidative process are known, the energy metabolism of nitrifying bacteria will remain obscure.

Nitrobacter oxidizes nitrite to nitrate in accordance with the following equation:

$$NO_2^- + \tfrac{1}{2} O_2 = NO_3^- \qquad \Delta F_{298}° = -18.0 \text{ kcal.} \tag{43}$$

From the free energies of formation of NO_2^- and NO_3^- (—28.5 kcal.), $\Delta F°_{298}$ for the reaction is —18.0 kcal. Note that the purely chemical reaction (as written) is independent of hydrogen ion concentration. Thus the free energy change for the reaction with the given species of ions will not change with pH. Somewhat less energy is available if oxygen is at a partial pressure of 0.2 atm., *viz.*, —17.5 kcal. It is also to be noted that the free energy value will change only slightly even with extreme changes in oxygen tension. For example, the free energy change associated with the conversion of a half mole of 0_2 at 0.01 atm. to 1.0 atm. at 25° is only

$$\Delta F_{298}° = 0.5\, RT \ln \frac{(P_2)}{(P_1)} = 0.5(1364) \log_{10} 10^2 \text{ cal.} = 1.36 \text{ kcal.} \tag{44}$$

Again, little is known as to the energy-coupling reactions or metabolic pathways in general in *Nitrobacter*. Biochemical information on the nitrifying bacteria has not been forthcoming primarily because adequate techniques are not yet available for the cultivation of these important organisms (*165*).

B. DENITRIFICATION

Denitrification is the term employed for the formation of gaseous nitrogen products from soils by microflora described by Gayon and Dupetit in 1886 (*170–172*). Nitrate or nitrite serves in place of atmospheric oxygen as an electron acceptor for certain facultative microorganisms. The reduced forms of nitrogen generally appear as the gaseous products, N_2, N_2O (nitrous oxide), or NO (nitric oxide).

Denitrification is effected by a large number of microorganisms in the genera (*171, 172*) *Pseudomonas, Micrococcus, Spirillum, Achromobacter,* and *Thiobaccillus.* All known denitrifying bacteria can grow with oxygen in absence of nitrate (*173*).

The only step in denitrifying organisms which is understood with any degree of clarity is the reduction of nitrate to nitrite catalyzed by nitrate reductase. This enzyme is discussed below in conjunction with nitrate reduction. The reactions in denitrifying organisms are thought to be related closely to those occurring in forms which reduce nitrate or nitrite as an assimilatory mechanism, as opposed to the employment of the latter compounds as mere electron acceptors for respiratory purposes.

In connection with nitrate reduction in general, and with denitrification in particular, it is to be noted that hydroxylamine (−1 oxidation level of nitrogen) is unstable with respect to decomposition into ammonium ion and nitrous oxide (*4*):

$$4\ NH_3OH^+ = N_2O + 2\ NH_4^+ + 2\ H^+ + 3\ H_2O \qquad (45)$$
$$\Delta F^\circ = -119 \text{ kcal.}$$

C. Nitrate and Nitrite Reduction

A large part of the nitrogenous matter in fungi and higher plants is derived from soil nitrate. The use of nitrates as fertilizers has long been recognized (*174*).

1. *Nitrate Reductase*

Nitrate reductase catalyzes the over-all reduction of nitrate to nitrite according to the following equation:

$$\text{TPNH (or DPNH)} + NO_3^- + H^+ = \text{TPN}^+ \text{ (or DPN}^+\text{)} + NO_2^- + H_2O \qquad (46)$$
$$\Delta F^{\circ\prime} = -38 \text{ kcal. } (\mathit{175, 176})$$

The occurrence of the enzyme is widespread in various microorganisms and plants [see McElroy and Glass (*164*)]. The enzyme is present, for example, in denitrifiers such as *Pseudomonas stutzeri, Bacillus subtilis, Escherichia coli* and *Micrococcus denitrificans;* in *Neurospora, Rhizobium* (a typical N-fixer), and the leaves of higher plants. Reduction of nitrate by a cell-free bacterial system was first demonstrated with an extract of *E. coli* (*177*).

In *Neurospora* preparations, FAD (or FMN) and molybdenum participate as electron carriers between TPNH and nitrate (*175, 178*) [TPNH → FAD (or FMN) → Mo → NO_3^-]. *Neurospora* nitrate reductase shows greater specificity for TPNH than for DPNH (*176*), while the enzyme from soybean nodules requires DPNH (*179*). Preparations from leaves of higher plants show less specificity, in that they can utilize either TPNH or DPNH (*180*).

On the basis of the participation of cytochromes as electron carriers, Sato (*181*) has tentatively grouped into three categories organisms carrying out nitrate reduction. Group I includes those organisms in which a cytochrome is held at some oxidized steady state during the reduction process. The steady state level of cytochrome in organisms of Group II is unaffected by the reduction process. Presumably cytochrome does not participate in the reduction in organisms of Group II; the reduction is mediated by molybdo-flavoproteins. Group III consists of organisms which do not contain cytochromes and hence must use the molybdo-flavin system (or some other). Members of the latter group have not been studied extensively. In general, those of Group I use nitrate primarily for respiratory purposes while those of Group II perform a true assimilation of nitrate and lack the ability to use nitrate as an electron acceptor. Organisms suspected of representing these types are tabulated in Table II. In some cases cytochromes and molybdo-flavin carriers may both participate in the reductive process.

TABLE II

TYPES OF NITRATE REDUCTION BY MICROORGANISMS[a]

Reduction type	Cytochromes	Organism	Electron carrier
I	Held at oxidized steady state during reduction	*E. coli* *Ps. stutzeri* *M. denitrificans* *Ps. denitrificans* *Staphylococcus aureus* *Corynebacterium diphtheriae*	Cytochrome
II	Steady state not affected during reduction	*N. crassa* NO_3-adapted: *B. subtilis* *Alcaligenes faecalis* *Azotobacter vinelandii*	Mo-flavin
III	None present	*Cl. welchii* anaerobes	?

[a] *cf.* Sato (*181*).

2. *Nitrite and Hydroxylamine Reductases*

Enzymes reducing nitrite and/or hydroxylamine have been studied in various species of *Bacillus* and in *E. coli*, *Neurospora*, and in leaves of higher plants (see Nason, reference *175*, p. 128). The reaction catalyzed by nitrite reductase is obscure in that it involves passage of nitrogen at the +3 oxidation level through an unknown intermediate at the +1 state. Hydroxylamine reductase catalyzes the reaction,

$$\text{TPNH (DPNH)} + NH_2OH + 2\,H^+ \rightarrow \text{TPN}^+ (\text{DPN}^+) + NH_4^+ + H_2O \qquad (47)$$

$$\Delta F^{\circ\prime} = -47 \text{ kcal. } (182)$$

Metalloflavins participate in the reaction. The physiological significance attached to hydroxylamine as an intermediate in assimilation of nitrogen compounds is discussed elsewhere (*164, 183–185*).

The green alga, *Ankistrodesmus braunii,* contains hydrogenase and can utilize molecular hydrogen to reduce nitrite to ammonia (*186*). Strains of *Chlorella,* not containing hydrogenase, can not use molecular hydrogen.

D. Nitrogen Fixation

Nitrogen fixation is the process whereby atmospheric nitrogen (N_2) is converted to N-containing organic compounds. Nitrogen fixation is effected by a variety of microorganisms (*164, 167, 183–185, 187, 188*): the free-living bacteria *Azotobacter* and *Clostridia;* photosynthetic bacteria, eg., *Rhodospirillum rubrum;* the blue-green algae of Nostocaceae; and the symbiont, *Rhizobium,* which infects the root nodules of legumes.

For some time it has been known that N^{15}-labeled nitrogen or ammonia is rapidly converted by various N-fixers to glutamic acid, and in lesser quantities to aspartic acid and alanine (*184*). The inference drawn from such studies was that ammonia represents the key intermediate in the reduction of nitrogen gas to amino nitrogen. The term "key intermediate" is defined as the end product of the nitrogen-fixing reactions and the initial product for nitrogen assimilation (*189*). If an intermediate other than ammonia is formed, it is rapidly converted to ammonia. The possible role of hydroxylamine as the key intermediate is discussed at length elsewhere (*183, 184*).

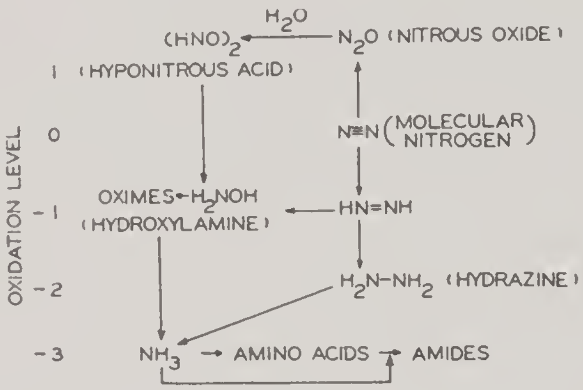


Fig. 3. Proposed pathways for ammonia formation by nitrogen fixation [adapted from Burris (*189*)].

In spite of recent advances in our knowledge of inorganic nitrogen metabolism, the initial biochemical event in preparing N_2 for subsequent reduction to lower oxidation level products is obscure.

The study of nitrogen fixation, and of inorganic nitrogen metabolism in general, has lagged, in part, because of the plethora of stable and unstable nitrogen compounds at various oxidation levels. Nitrogen compounds exist at all oxidation levels from −3 to +5. Some hypothetical intermediates decompose spontaneously, and some are highly reactive (hydrazine, hydroxylamine). Our present state of knowledge of chemical transformations in nitrogen fixation is summarized (*189*) in Fig. 3. Molecular nitrogen could be reduced successively to ammonia via HN=NH and hydrazine (enzyme-bound?). On the other hand, nitrogen could first be oxidized to nitrous oxide, and via hyponitrous acid could then be converted into hydroxylamine, oximes, and finally ammonia. Conversion of nitrous oxide to hyponitrous acid poses a serious thermodynamic impasse (*4*) (+ 45 kcal.) in this alternate scheme.

It is of interest to note that the over-all reduction of N_2 to NH_4^+ by 3 moles of a reduced pyridine nucleotide is thermodynamically favorable at pH 7:

$$N_2 + 3\ DPNH + 5\ H^+ = 2\ NH_4^+ + 3\ DPN^+ \qquad (47a)$$
$$\Delta F_{298}{}^{\circ\prime} = -6\ \text{kcal.}$$

Reduction of molecular nitrogen to hydroxylamine in a 2-electron step would seem to be virtually impossible with known cellular reducing agents. The half-reaction

$$N_2 + 2\ H^+ + 2\ H_2O + 2\ e^- = 2\ H_2NOH \qquad (47b)$$

has associated with it a standard free energy change at pH 7 of +121 kcal. Coupling this half-reaction with that of DPNH → DPN ($\Delta F_{298}{}^{\circ\prime}$ = −14.7 kcal.) yields the following reaction with a highly unfavorable free energy change:

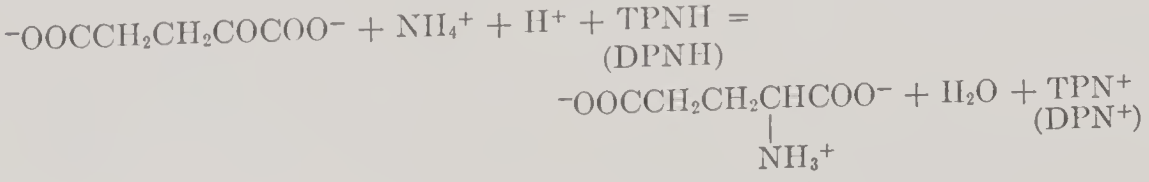
$$H^+ + N_2 + DPNH + 2\ H_2O = 2\ H_2NOH + DPN^+ \qquad (47c)$$
$$\Delta F_{298}{}^{\circ\prime} = +106\ \text{kcal.}$$

The older classifications of nitrification, denitrification, nitrate reduction, and nitrogen fixation will eventually give way as the enzymology of these processes is worked out. There is already more than the hint that these arbitrary categories have much in common. Such processes seem to be but variants of a more general theme, the variants contributing to the economy of the diverse forms which manipulate inorganic nitrogen compounds.

VI. Systems Utilizing Ammonia

A. Glutamic Dehydrogenase

The most general mechanism for the assimilation of ammonia appears to be the reductive amination of α-ketoglutaric acid to glutamic acid by glutamic acid dehydrogenase:



$$^-OOCCH_2CH_2COCOO^- + NH_4^+ + H^+ + \underset{(DPNH)}{TPNH} = \underset{\substack{|\\ NH_3^+}}{^-OOCCH_2CH_2CHCOO^-} + H_2O + \underset{(DPN^+)}{TPN^+} \qquad (48)$$

$$\Delta F_{300}{}^{\circ\prime} = -17.8\ \text{kcal. } (\mathit{190})$$

As early as 1910, Knoop (*191*), and Embden and Schmitz (*192*) demonstrated that α-keto acids were reductively aminated to the corresponding amino acids by animals. Such amino acids are now known to arise secondarily from corresponding α-keto acids and glutamic acid by transamination systems present throughout the plant and animal kingdoms (*56a, 57, 193*). The uptake of ammonia is closely related to the operation (partially or totally) of the tricarboxylic acid cycle which is practically ubiquitous in nature (*194*). As an intermediate of this cycle, α-ketoglutarate can be readily supplied from carbohydrate precursors, leading to synthesis of glutamic acid and a host of other amino acids by transamination.

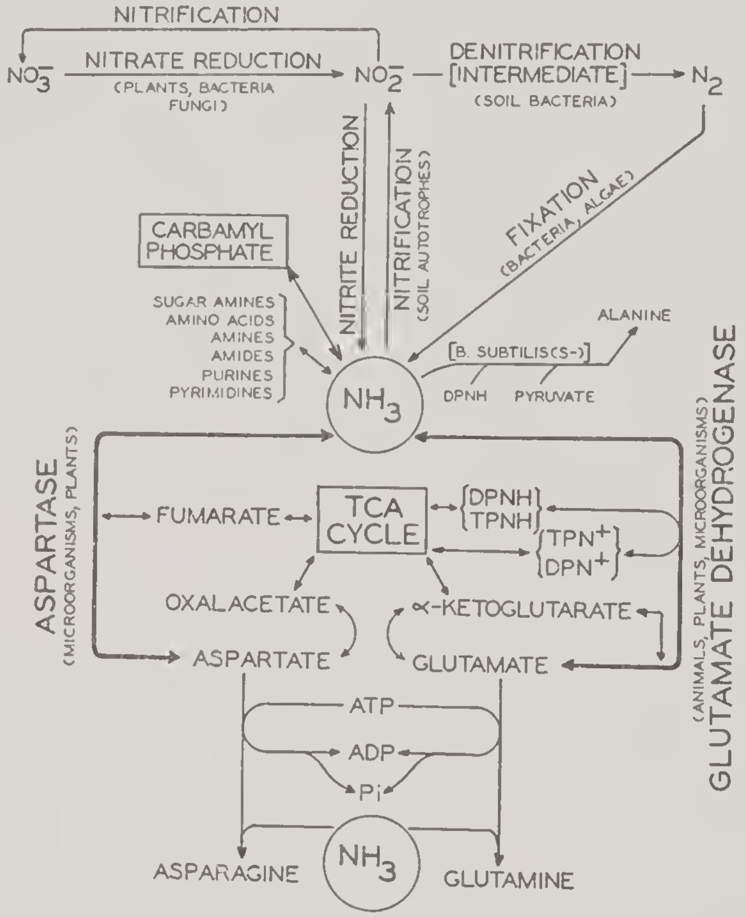


FIG. 4. Composite scheme for ammonia formation and utilization.

Reductive amination, directly, of α-keto acids other than α-ketoglutarate has received experimental support recently (cf. Section IV,A,1). Such reactions have been proposed from time to time (*195, 196*).

Glutamic dehydrogenase has been found (*183, 197*) in green plants (*198–203*), animals (*190, 204–213*), yeast (*214, 215*), *E. coli* (*216*), and *Neurospora* (*217–219*). The apoenzyme is active only toward the L-form of glutamic acid. The nature of the pyridine nucleotide acting as coenzyme depends upon the source of the apoenzyme (*215*): in higher plants, DPN; in yeast and bacteria, TPN; and in higher animals, either DPN or TPN. Information as to the occurrence of this enzyme in blue-green algae and the type of coenzyme employed would further our knowledge of the relationship of these primitive plants to bacteria and higher plants. Blue-green algae are thought to be closely related to bacteria (*220, 221*). It thus seems likely that TPN would serve as the coenzyme in Cyanophyceae.

The change in free energy associated with the reductive amination of α-ketoglutarate was determined from kinetic and equilibrium studies with crystalline beef liver glutamic dehydrogenase (*190*). The large negative value indicates that the synthesis of glutamic acid is favored although the reaction is reversible.

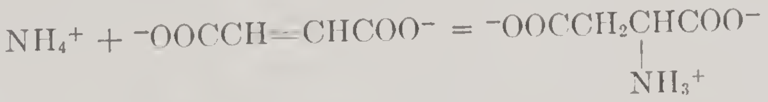
The enzymatic rate with the beef liver enzyme depends upon the pyridine nucleotide employed. Deamino-DPN^+ and TPN^+ yield only 60% and 35%, respectively, of the rate achieved with DPN^+.

Mutant strains of *Neurospora* have been obtained which are deficient in glutamic dehydrogenase (*217–219*). Hence, these, unlike the wild-type strains, are unable to synthesize the amino groups of a large number of amino acids from ammonia. The genetic control of this enzyme has been well established.

B. ASPARTASE

Aspartase (*222*), which catalyzes the reversible addition of ammonia to fumaric acid, is widely distributed throughout bacterial groups (*223–232*), and may be present in yeast (*233*) and in the mold, *Penicillium notatum* (*222*). Certain plant seedlings, notably those of *Phaseolus mungo, Cicer arietinum,* and *Pisum sativum,* possess aspartase activity (*234*). Activity of this enzyme is weak or nonexistent in higher plants. The enzyme is probably absent in tissues of higher animals; little, if any, information is available on the occurrence of the enzyme in insects, protozoa, or algae. The enzyme appears to be typically bacterial.

If the reaction catalyzed by aspartase is formulated as



$$NH_4^+ + {}^-OOCCH{=}CHCOO^- = {}^-OOCCH_2\underset{\substack{|\\ NH_3^+}}{C}HCOO^- \qquad (49)$$

$$\Delta F^{\circ\prime} = -2.7 \text{ kcal. (calculated from equilibrium constant) } (73).$$

The reaction is reversible and synthesis of aspartic acid is favored. Fumaric acid participates in *reversible* reactions of the tricarboxylic acid cycle, and hence a greater variety of reactions exist for provision of an acceptor for ammonia in the aspartase reaction than in the glutamic dehydrogenase reaction.

C. ROLE OF GLUTAMIC AND ASPARTIC ACIDS IN METABOLISM

Both glutamic and aspartic acids occupy a central role as carriers for nitrogen at the oxidation level of —3 (ammonia, amine, amide). Nitrogen at higher oxidation levels such as in nitrate and molecular nitrogen are brought to this level through the processes of nitrate reduction and nitrogen fixation already discussed (Section V). Assimilation of ammonia via aspartase in bacteria and plants, and by glutamic dehydrogenase in animals and a variety of organisms, places these dicarboxylic amino acids at the hub of intermediary nitrogen metabolism. Other amino acids can be derived from glutamate and aspartate and the keto acids by mediation of the ubiquitous (*9, 57, 193, 235*) enzymes catalyzing transamination reactions.

In addition to its assimilatory role, glutamic acid participates in reaction schemes (*9, 10, 56a, 236*) involving histidine, γ-aminobutyric acid, glycine and cysteine (glutathione synthesis), hydroxyproline, proline, ornithine, folic acid, and detoxication products.

Aspartic acid is a specific amino donor for one of the amide groups of urea (*237*). The amino acid amides, glutamine and asparagine, are important chemical reservoirs for ammonia (*106–108, 238, 239*).

D. GLUTAMINE SYNTHETASE AND TRANSFERASES

The ubiquitous occurrence (*104, 108, 238, 240*) of glutamine in diverse organisms signifies a fundamental role for the ω-amide of glutamic acid. A voluminous literature exists on the distribution of glutamine in nature, and subsequent to the review by Meister (*108*), its occurrence is reported in toad embryos (*241*), lobsters (*242*), mushrooms (*243*), alder and pea plants (*244*), spinach leaves (*245*), etc., and unfermented beer wort (*246*).

Glutamine has long been recognized (along with asparagine) as a reservoir of ammonia nitrogen. Teleologically, glutamine may be regarded as a compound synthesized as a protective mechanism to maintain a low concentration of ammonia in tissues. Kinetic studies of N^{15}-ammonia metabolism in intact rats show in striking fashion the efficiency of glutamine synthesis (*246a*). As indicated in Section VII, the urea cycle also serves to maintain, at least in animals, a low concentration of ammonia through its removal in the form of an excretory product (urea).

1. *Glutamine Synthetase*

The early studies of Krebs (*247*) demonstrated the ability of various tissues from mammals and birds to effect the synthesis of glutamine enzyme depends upon the source of the apoenzyme (*215*): in higher plants, DPN; in yeast and bacteria, TPN; and in higher animals, either DPN or TPN. Information as to the occurrence of this enzyme in blue-green algae and the type of coenzyme employed would further our knowledge of the relationship of these primitive plants to bacteria and higher plants. Blue-green algae are thought to be closely related to bacteria (*220, 221*). It thus seems likely that TPN would serve as the coenzyme in Cyanophyceae.

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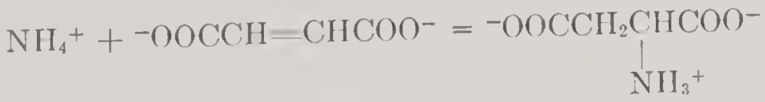

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1. *Glutamine Synthetase*

The early studies of Krebs (*247*) demonstrated the ability of various tissues from mammals and birds to effect the synthesis of glutamine from ammonium glutamate. Subsequent studies by Speck (*248–250*), Leuthardt and Bujard (*251*), and others (*252–258*) with cell-free extracts and purified enzymes permit the reversible (*258*), synthetic reaction to be formulated as

$$\text{Glutamate}^{\overset{\mp}{-}} + \text{ATP} + \text{NH}_4^+ = \text{glutamine}^{\mp} + \text{ADP} + \text{P}_i. \qquad (50)$$

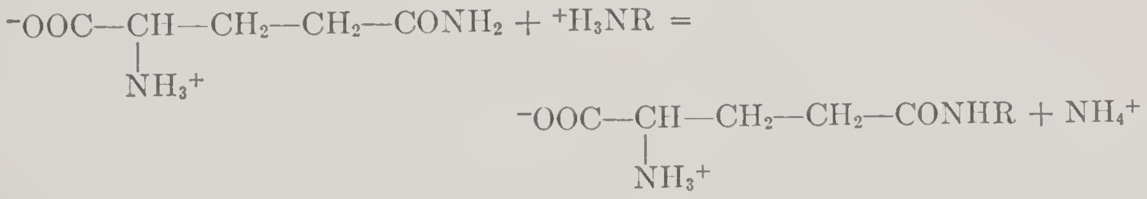
$\Delta F^{\circ\prime} = -4.3$ kcal. (calculated from the thermodynamic equilibrium constant) (*258*)

Glutamine synthetase prepared from higher vertebrate tissues (*248–253, 258*), *Staphylococcus aureus* (*252*), yeast (*252*), peas (*254, 257*), or lupine seedlings (*Lupinus albus*) (*252, 255*), is activated by Mg^{2+} or to a lesser extent by Mn^{2+}. Cobalt is also known to activate the animal and plant enzymes (*256*).

Hydroxylamine or hydrazine can replace ammonia in the synthetic reaction (*250, 252–254*).

2. *Glutamine Transferases*

Enzyme preparations carrying out the synthesis of glutamine from glutamic acid and ammonia are capable of catalyzing the exchange of the amide group of glutamine with hydroxylamine, hydrazine, or $N^{15}H_3$ (*259–261*).



$$^{-}\text{OOC—}\underset{\text{NH}_3^+}{\underset{|}{\text{CH}}}\text{—CH}_2\text{—CH}_2\text{—CONH}_2 + {}^{+}\text{H}_3\text{NR} = {}^{-}\text{OOC—}\underset{\text{NH}_3^+}{\underset{|}{\text{CH}}}\text{—CH}_2\text{—CH}_2\text{—CONHR} + \text{NH}_4^+ \qquad (51)$$

(R = —NH_2, —OH, or —H)

The usual glutamotransferase prepared from vertebrates or bacteria requires Mn^{2+}, phosphate (or arsenate), and the adenine nucleotides, ATP or ADP, for maximal activity. These same requirements hold for the transferases from pumpkin seedlings, *Cucurbita pepo* var. Sugar Pumpkin (*261–263*), and *Neurospora* (*264*) except that these are *completely* dependent upon nucleotides for activity while the transferases from vertebrates or bacteria are not. The pumpkin enzyme is not inhibited by iodoacetate and hence differs from the animal enzyme in this respect. The situation is complicated by the fact that manganese-independent glutamotransferase and aspartotransferase are known to occur in bacteria (*265*).

Bacterial Mn-independent transferase may be completely separated from synthetase activity. This has not been realized with the Mn-dependent enzyme, regardless of source. Only Cu^{2+} activates the Mn-independent bacterial glutamotransferase. It has a pH optimum of 8 while those from sheep brain, pigeon liver, or *P. vulgaris*, and requiring Mn^{2+}, exhibit their optima at 5.5 (*266*). Recent findings suggest that synthesis of amides, hydrolysis, and transfer reactions may involve separate enzymes in bacteria (*267*).

3. *Mechanism of Glutamine Synthesis*

In the presence of P^{32}-orthophosphate, ATP, and Mg^{2+}, the glutamine synthetase from peas slowly catalyzes an exchange reaction leading to incorporation of isotope into ATP (*268*). The net incorporation is enhanced markedly by the presence of glutamate and is depressed significantly by ammonia. The results are consistent with a series of steps such as shown (Reactions 52a, b, c).

$$\text{E} + \text{ATP} \rightleftharpoons \text{E-P} + \text{ADP} \tag{52a}$$
$$\text{E-P} + \text{Glutamate} \rightleftharpoons \text{E-Glutamate} + P_i \tag{52b}$$
$$\text{E-Glutamate} + NH_3 \rightleftharpoons \text{E} + \text{Glutamine} \tag{52c}$$

In this formulation of the glutamine synthetase reaction, glutamate would be essential for step b to proceed, enabling E-glutamate to be formed. In the back reaction, E-glutamate + P_i^{32} would yield E–P^{32} and thus ATP^{32}. NH_3 would diminish exchange by lowering the concentration of E-glutamate. Such a formulation indicates that glutamine synthetase would exhibit glutamotransferase activity through participation of step c only. Alternative formulations are possible.

Further discussion of glutamosynthetase and transferase activities and their reaction mechanisms is beyond the scope of the present chapter. Studies on the reaction mechanism have been reported recently (*269*). The ability to synthesize glutamine is a widespread property of cells, and the amide nitrogen can be made available for reactions involving ammonia through hydrolytic cleavage by glutaminase. The part glutamine may play in transamidation reactions is still obscure.

E. Asparagine Synthetase

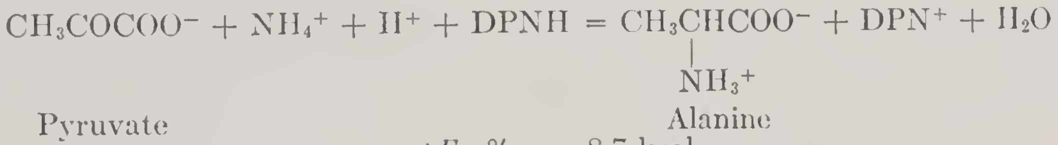
Asparagine synthesis with liver extracts by transamination between α-ketosuccinamic acid and a variety of L-amino acids has been reported (*270*). Synthesis from aspartic acid, ammonia and ATP in wheat germ extracts has been demonstrated (*271*). Extracts of lupine seedlings (which accumulate as much as 25% of their dry weight as asparagine (*272*)) were also active but required no ATP. The properties of the enzyme preparations were sufficiently different from the glutamine synthetase systems to suggest an independent pathway.

F. Carbamyl Phosphate Synthetase and Carbamate Kinase

Assimilation of ammonia via aspartase or glutamic acid dehydrogenase is complemented by another ammonia fixation reaction which occurs in liver of true ureotelic animals and to a slight but significant extent in intestinal mucosa (*273*). Because of the importance of carbamyl phosphate synthetase for the production of urea and its relation to the carbamate kinase occurring in many bacteria, discussion of these enzymes appears in the next section. The relationship of ammonia fixation in animal tissues, as carried out by carbamyl phosphate synthetase, to the intermediary metabolism of ammonia, in general, has only recently been appreciated. Its relationship to the enzyme of bacterial origin presents an interesting commentary on the comparative aspects of the reaction(s) catalyzed by these enzymes.

G. MISCELLANEOUS AMMONIA FIXATION REACTIONS

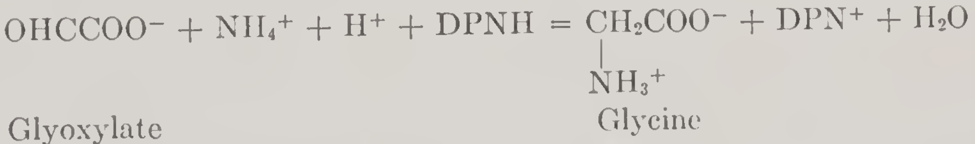
Several new ammonia fixation reactions have been described recently which appear to be confined to microorganisms. One of these is catalyzed by enzymes found in a mutant strain of *B. subtilis* (*31*) and in the $H_{37}Ra$ strain of *Mycobacterium tuberculosis* var. *hominis* (*31a*). Pyruvate is reductively aminated to alanine by DPNH:



$$CH_3COCOO^- + NH_4^+ + H^+ + DPNH = CH_3\underset{\substack{|\\ NH_3^+}}{C}HCOO^- + DPN^+ + H_2O \qquad (52d)$$

$\Delta F_{298}{}^{\circ\prime} = -8.7$ kcal.

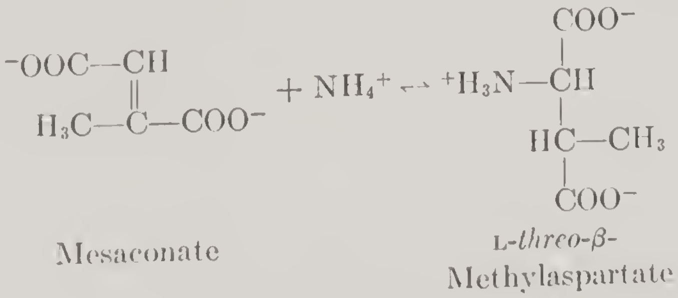
The reaction is reversible. *M. tuberculosis* var. *hominis* also possesses an enzyme which converts glyoxylate to glycine irreversibly (*273a*):



$$OHCCOO^- + NH_4^+ + H^+ + DPNH = \underset{\substack{|\\ NH_3^+}}{CH_2}COO^- + DPN^+ + H_2O \qquad (52e)$$

$\Delta F_{298}{}^{\circ\prime} = -11$ kcal.

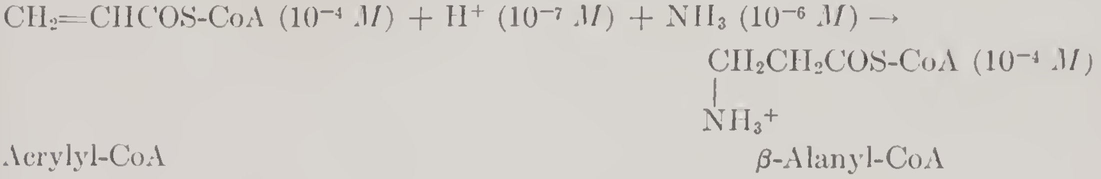
The two reactions described above are analogous to the glutamic acid dehydrogenase reaction (Eq. 48) in that all these represent reductive aminations. Two other reactions have been described which are analogous to the aspartase reaction (Eq. 49) in that they involve an addition of ammonia across a double bond. *Clostridium tetanomorphum*, an organism which rapidly ferments glutamate, possesses the enzyme β-methyl aspartase which catalyzes the reversible reaction (*273b*) shown (Eq. 52f).



$$\begin{array}{l} {}^-OOC-CH \\ \qquad\quad \| \\ H_3C-C-COO^- \end{array} + NH_4^+ \rightleftharpoons \begin{array}{l} \qquad\quad COO^- \\ \qquad\quad | \\ {}^+H_3N-CH \\ \qquad\quad | \\ \qquad\quad HC-CH_3 \\ \qquad\quad | \\ \qquad\quad COO^- \end{array} \qquad (52f)$$

$\Delta F^{\circ\prime} = -0.85$ kcal.

Clostridium propionicum ferments β-alanine to propionate, acetate, carbon dioxide and ammonia, and extracts of this organism have been shown to catalyze the following reaction (*273c*):



$$\underset{\text{Acrylyl-CoA}}{CH_2{=}CHCOS\text{-}CoA\ (10^{-4}\ M)} + H^+\ (10^{-7}\ M) + NH_3\ (10^{-6}\ M) \rightarrow \underset{\text{β-Alanyl-CoA}}{\underset{NH_3^+}{CH_2}CH_2COS\text{-}CoA\ (10^{-4}\ M)} \quad (52g)$$

$$\Delta F_{298} \simeq -0.6 \text{ kcal.}$$

Free, uncharged ammonia is thought to participate as the active species in the exergonic reaction. The free energy datum was obtained from equilibrium studies in which acrylyl pantetheine was employed as substrate. Original data have been used for the above calculation.

VII. Biosynthesis of Urea

The synthesis of urea has been identified with two milestones in the history of biochemistry. Wohler's synthesis of urea from ammonium cyanate in 1828 is considered to be the event which signaled the birth of biochemistry (originally called organic chemistry). The demonstration of the cyclic nature of the biosynthesis of urea in tissue slices by Krebs and Henseleit in 1932 (*274*) represented a major advance in initiating the phase of modern biochemistry which is concerned with endergonic biosynthetic mechanisms.

The biosynthesis of urea from ammonia and carbon dioxide was considered to be, until 1946, a unique property of organized and surviving liver cells (*275*). At this time, because of the advances made in our understanding of the role of ATP as an energy-yielding component for endergonic systems, it was possible to demonstrate the synthesis of urea from citrulline in cell-free systems (*276*). Following this, arginine synthesis from citrulline (*277–283*) and citrulline synthesis from ornithine (*279, 284–287*) were demonstrated in cell-free systems. As a result of these early studies, the nature of the individual enzymatic steps and the nature of the energy-coupling reactions became known.

While the main over-all enzymatic steps of the urea cycle were suggested from the formulation by Krebs and Henseleit, this formulation did not provide information on the nature of the individual steps. A clue as to the enzymatic complexity of the system was provided by the study of Srb and Horowitz (*288*) who were able to demonstrate the operation of an ornithine cycle in *Neurospora crassa* and further to demonstrate, in a variety of mutants, intermediate requirements. Such requirements suggested the existence of a number of discrete enzymatic steps, all of which were present in the wild type but not in the different mutants. This apparently unrelated study involving a microorganism served to orient the enzymatic approach to the urea cycle in animal preparations. Thus we have here another example of the value of a comparative biochemical approach.

The early studies with cell-free systems indicated the existence of the following steps in the urea cycle:

$$\text{Ornithine} + CO_2 + NH_3 \rightarrow \text{Citrulline} \tag{53}$$
$$\text{Citrulline} + \text{Amino-donor} \rightarrow \text{Arginine} \tag{54}$$
$$\text{Arginine} \rightarrow \text{Ornithine} + \text{Urea} \tag{55}$$

Reaction 55, catalyzed by arginase, has long been known as a mechanism for urea production. High arginase activity in livers of ureotelic animals has been recognized since the early studies of Clementi (see *289, 290*).

The over-all reaction represented by Reaction 53 leading to citrulline synthesis in time was resolved into 2 enzymatic steps (*291–296*):

$$CO_2 + NH_3 + \text{ATP} (+ \text{Glutamyl derivative}) \rightarrow \text{Intermediate} \tag{56}$$
$$\text{Intermediate} + \text{Ornithine} \rightarrow \text{Citrulline} \tag{57}$$

While the intermediate isolated from animal systems was originally considered to be a glutamate derivative (*297*), it is now clear that this is in fact carbamyl phosphate (*298–300*). Bacterial systems do not require a glutamate derivative for carbamyl phosphate synthesis (*298, 301*). A further difference between bacterial and animal systems is the requirement of 2 moles of ATP per mole of carbamyl phosphate formed in the animal system (*302*). From present knowledge it appears that Reaction 57 represents a single enzymatic transcarbamylation reaction (*95, 295, 296, 303, 303a*).

The over-all step shown in Reaction (54), which was originally considered to be a simple transimination reaction (*304*), has been resolved into 2 enzymatic steps (*237, 305–312*).

$$\text{Citrulline} + \text{Aspartate} + \text{ATP} \rightarrow \text{Argininosuccinate} \tag{58}$$
$$\text{Argininosuccinate} \rightarrow \text{Arginine} + \text{Fumarate} \tag{59}$$

The subsequent development and current status of the individual enzymatic steps are related below.

It should be emphasized that the biosynthesis of urea represents a unique biological system in that it involves, as shown in Reaction 56, the *primary fixation* of *both carbon dioxide and ammonia*. While the importance of this has been emphasized chiefly in relation to the highly specialized function of urea biosynthesis, it should be pointed out that the formation of carbamyl phosphate may be of more fundamental importance from the standpoint of biochemical evolution. Thus not only does carbamyl phosphate represent an energy-rich compound formed as a result of the primary fixation of carbon dioxide and ammonia but it also represents a component of two different systems, one leading to the synthesis of an amino acid, arginine, and the other to the synthesis of pyrimidines. Pyrimidine synthesis in relation to urea synthesis is discussed in Section VII,E.

A. Enzymatic Steps and Nature of the Cycle

1. *Carbamyl Phosphate Synthetase and Carbamyl Phosphate Kinase*

a. Animals. The first step(s) in the biosynthesis of urea in animals may be regarded as the formation of carbamyl phosphate (*298–300*). The over-all reaction, catalyzed by carbamyl phosphate synthetase may be formulated (*291–296, 302*)

$$NH_4^+ + HCO_3^- + 2\ ATP \xrightarrow[\text{acyl glutamate}]{Mg^{2+}} H_2NCO \sim P + 2\ ADP + P_i \qquad (60)$$

$$\Delta F^{\circ\prime} = -2\ \text{kcal. (estimated)}$$

The enzyme occurs in the particulate fraction of the livers of vertebrates such as the rat, cow, rabbit, monkey, frog, and turtle (*295*). It is notably absent in avian liver. Studies with the crude enzyme demonstrated the participation of ATP and established that Mg^{2+} and an *N*-acyl derivative of glutamic acid were required as cofactors. Typically, such compounds as formyl-, acetyl-, chloroacetyl-, and carbamyl glutamate are active in the system (*295*). *N*-Acetyl glutamate, the most active of the glutamate derivatives, has been isolated from fresh liver (3 parts per million) (*313*); it has been proposed as an intermediate in ornithine synthesis from glutamate in *Escherichia coli* (*314, 315*).

As yet no definitive role can be assigned for the action of the glutamate derivatives in the synthesis of carbamyl phosphate. However, present evidence from the authors' laboratory (unpublished) suggest that it is required in the CO_2^- fixation step (see Fig. 5). Acylation of the amino group of glutamic acid decreases the basicity of the amino nitrogen, and this may be related to the role of acyl derivatives of glutamic acid as coenzymes. In a study of the mechanism of the reaction, it was found that neither the oxygen of water nor of the terminal phosphate of ATP was incorporated into *N*-acetyl glutamate during the synthesis of carbamyl phosphate (*315a*).

Carbamyl phosphate synthetase, prepared from the liver of the bullfrog *Rana catesbeiana*, has been brought to a high state of purity (*315b*). The purified enzyme shows the same requirements as the crude preparations, but with an absolute requirement for an *N*-acyl glutamate cofactor. It has now been established that 2 moles of ATP are required for the synthesis of 1 mole of carbamyl phosphate by both mammalian (*302*) and amphibian (*315b*) enzymes.

Only a few studies have been made on the occurrence of the reaction phylogenetically below the level of Vertebrata in the animal kingdom. Carbamyl phosphate synthetase could not be demonstrated in a single specimen of *Limulus*. Cell-free extracts of the ciliated protozoan, *Tetrahymena pyriformis* S. were reported to be capable of synthesizing citrulline from ornithine, ATP, ammonia, and bicarbonate under conditions similar to those employed with rat liver preparations (*316*). However, this finding could not be confirmed (*316a*). Preliminary findings indicate the presence of the enzyme (or one similar to the bacterial enzyme—see below) in *Neurospora* (*317*).

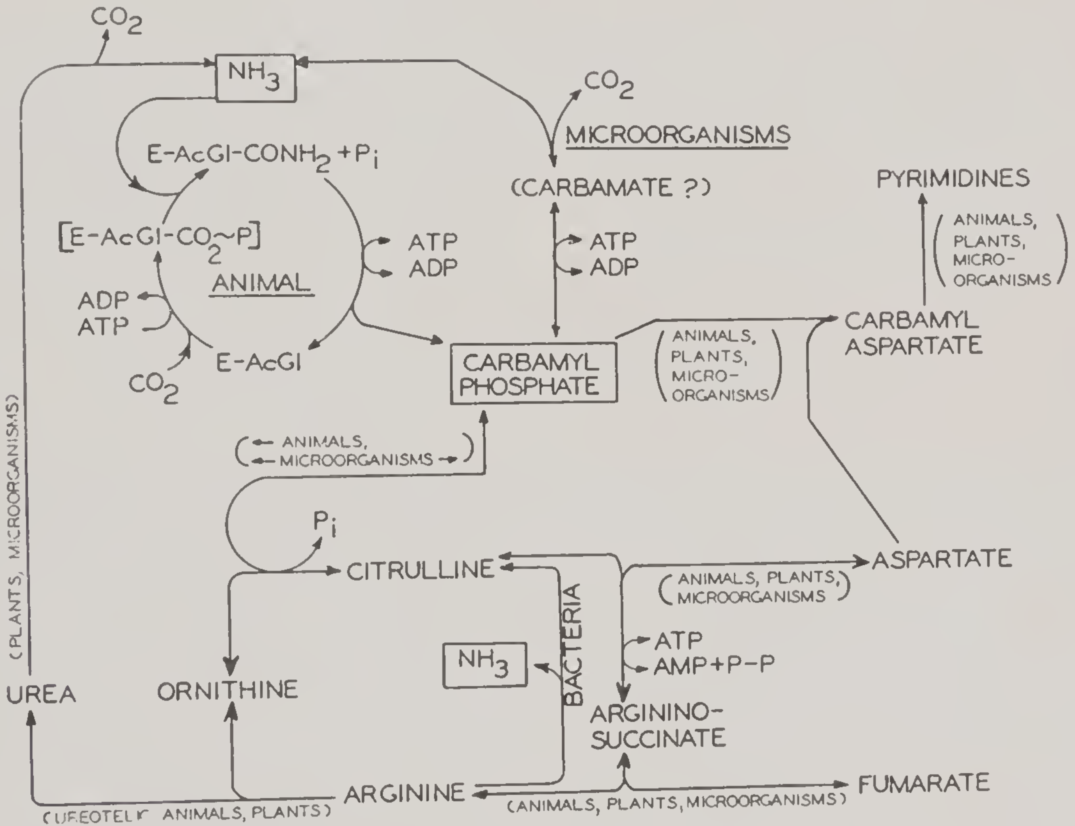


FIG. 5. Metabolic interrelationship of carbamyl phosphate, urea cycle, and pyrimidine synthesis.

b. Bacteria. Streptococcus faecalis and other bacteria (*83–90, 318–320*) possess an enzyme, carbamate kinase, which is related to the carbamyl phosphate synthetase found in nonavian, vertebrate liver. The presence of this kinase was deduced from observations on the substrate-level generation of ATP which was linked to the anaerobic conversion of citrulline to ornithine (*83–85, 318–320*). The bacterial enzyme catalyzes the *reversible* synthesis of carbamyl phosphate:

$$H_2NCO\sim P + ADP + H_2O \overset{Mg^{2+}}{=} NH_4^+ + HCO_3^- + ATP \qquad (61)$$

The bacterial enzyme differs from that in liver in that the former requires only one mole of ATP for synthesis of carbamyl phosphate and is not dependent upon an *N*-acyl derivative of glutamic acid. The easy reversibility of the reaction in which only 1 mole of adenine nucleotide is employed suggests that the free energy of hydrolysis of ATP and of carbamyl phosphate (to carbamic acid + P_i) are nearly the same.

Synthesis of ATP is coupled with the conversion of citrulline according to reactions shown (62–64).

$$\text{Citrulline} + P_i = \text{Ornithine} + \text{Carbamyl phosphate} \qquad (62)$$

$$\text{Carbamyl phosphate} + ADP + H_2O = ATP + NH_4^+ + HCO_3^- \qquad (63)$$

$$\text{Sum:}\quad \text{Citrulline} + ADP + P_i + H_2O = ATP + \text{Ornithine} + HCO_3^- + NH_4^+ \qquad (64$$

Reaction 64 is an unusual instance of synthesis of ATP from ADP and P_i coupled to a substrate reaction not involving an oxidation. This represents the citrulline phosphorylase reaction (see Section IV,B,7). A pseudomonad isolated from garden soil generates ATP from the coupled conversion of L-citrulline to ornithine, and in addition this bacterium grows on citrulline as a sole source of carbon and nitrogen (*89*). From a comparative standpoint, this organism is of great interest. For example, one would like to know what proportion of its total energy requirement is met by the nonoxidative generation of ATP.

The bacterial enzyme which synthesizes carbamyl phosphate (reverse of Eq. 61) has a lower affinity for ammonia than does the mammalian enzyme. Presumably, the bacterial enzyme phosphorylates *carbamic acid* which is in equilibrium with ammonia and bicarbonate:

$$NH_4^+ + HCO_3^- \rightleftharpoons H_2NCOOH + H_2O \qquad (65)$$
$$\Delta F^\circ \simeq 6 \text{ kcal. (est.)}$$

The additional ATP required with the mammalian (or amphibian) enzyme can be considered to provide the energy to overcome Reaction 65, enabling it to pick up ammonia at much lower concentrations than is the case with the bacterial enzyme.

c. Algae. The Cyanophyceae, or blue-green algae, contain relatively large amounts of citrulline and are able to fix large amounts of $C^{14}O_2$ into citrulline during photosynthesis (*321*). Members of the Chlorophyceae (green algae), on the other hand, have lesser amounts of citrulline and carry out this reaction to a limited extent. Ninhydrin treatment of the C^{14}-citrulline (which removes the carboxyl carbon) does not decrease the C^{14} content appreciably; it is likely that the isotope resides exclusively in the carbamyl ($H_2NCO—$) group. This finding is of great interest, for the blue-green algae may be able to carry out the coupled synthesis of ATP from citrulline just discussed. More recently it has been shown that cell-free extracts of the blue-green alga, *Nostoc muscorum*, in the presence of ATP, Mg^{2+}, NH_4^+, ornithine, and partially purified beef liver ornithine transcarbamylase, incorporate C^{14}-labeled bicarbonate into a compound which corresponds chromatographically to citrulline (*321a*). Partial degradation of the radioactive citrulline suggests that the major portion of the C^{14} resided in the ureido carbon. Exogenous *N*-acetyl glutamate was not required; thus the enzyme appears to be of the bacterial type.

In the blue-green algae, energy produced by photosynthetic reactions could be stored in citrulline to be used when required for endergonic reactions during nonphotosynthetic periods. Conceivably, citrulline could act as a high-energy reservoir, maintaining the ATP concentration within reasonable limits.

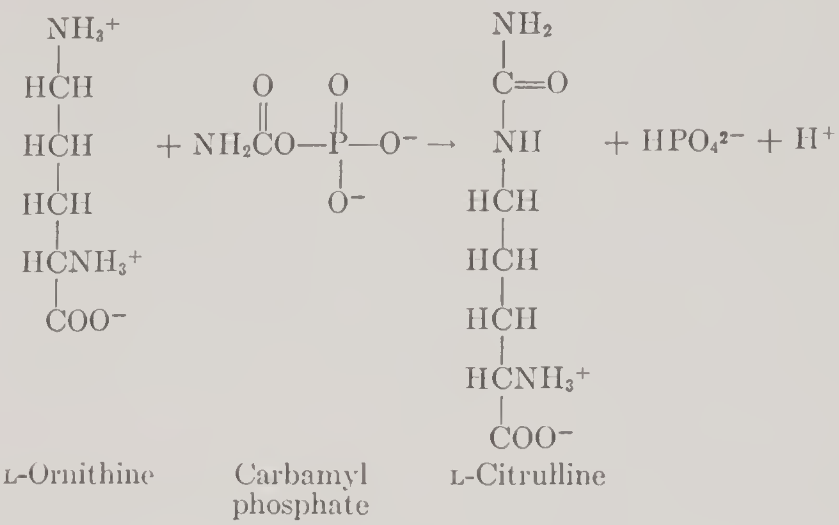
d. Yeast. Intact cells of *Saccharomyces cerevisiae* incorporate $C^{14}O_2$ into the ureido group of citrulline (*321b*), suggesting thereby that the yeast possesses both a carbamyl phosphate synthesizing enzyme and an ornithine transcarbamylase. Only limited studies have been carried out on such enzymes in this organism. It would be of interest to determine if the carbamyl phosphate synthesizing enzyme is of the animal or bacterial type.

e. Higher Plants. While it has been known for some time that citrulline occurs in higher plants, only recently has the pathway for its synthesis been demonstrated. Mitochondria prepared from the legume, *Phaseolus aureus* (mung bean), have been shown to incorporate $C^{14}O_2$ into citrulline via carbamyl phosphate (*321c*). Exogenous *N*-acetyl glutamate had no effect on citrulline synthesis; hence, the carbamyl phosphate synthesizing enzyme is presumably of the bacterial type. One may anticipate that more intensive studies on arginine synthesis in higher plants will be forthcoming now that the initial ammonia fixation reaction in the enzymatic sequence has been demonstrated.

2. *Ornithine Transcarbamylase*

Mammalian liver contains an enzyme, ornithine transcarbamylase, which catalyzes the synthesis of citrulline from ornithine and carbamyl phosphate (*95, 291–296, 303*) (Reaction 66).

Reaction 66 is the reverse of Reaction 62 which is concerned with the generation of ATP from citrulline and inorganic phosphate as occurs in *S. faecalis*. The mammalian enzyme does not catalyze the reaction reversibly to any appreciable extent (*95*). Ornithine, however, may



$$\begin{array}{c} NH_3^+ \\ | \\ HCH \\ | \\ HCH \\ | \\ HCH \\ | \\ HCNH_3^+ \\ | \\ COO^- \end{array} + NH_2\overset{\overset{\Large O}{\|}}{C}-\overset{\overset{\Large O}{\|}}{\underset{\underset{\Large O^-}{|}}{P}}-O^- \rightarrow \begin{array}{c} NH_2 \\ | \\ C{=}O \\ | \\ NH \\ | \\ HCH \\ | \\ HCH \\ | \\ HCH \\ | \\ HCNH_3^+ \\ | \\ COO^- \end{array} + HPO_4^{2-} + H^+ \qquad (66)$$

L-Ornithine   Carbamyl phosphate   L-Citrulline

$\Delta F_{310}°\ (\text{pH}8) < 0$

be generated from citrulline by arsenolysis or phosphorolysis according to the following reaction (*92, 95, 320*):

$$\text{Citrulline}^{\pm} + H^+ + 2H_2O \xrightarrow[\text{or Phosphate}]{\text{Arsenate}} \text{ornithine}^{\pm} + NH_4^+ + HCO_3^- \qquad (67)$$

$$\Delta F° < 0$$

Reaction 67 takes place with either the bacterial or mammalian enzymes. No energy is available for useful work from this reaction.

Ornithine transcarbamylase is absent from avian liver. A comparison of enzyme activities in the liver of various species is given in Table V. The enzyme has a high degree of substrate specificity. Details of partial purification of the mammalian enzyme and of its properties appear elsewhere (*95, 303*). Purification and some properties of the enzyme from *Streptococcus lactis* 8039 have been reported (*321d*).

The unidirectional catalysis of Reaction 66 by the liver enzyme indicates that it probably evolved or became so modified as to serve the economy of those organisms which form urea via the urea cycle. While reversibility of Reaction 66 is thermodynamically possible, operation of the urea cycle in a clockwise direction (see Fig. 5) is maintained, in part, by failure of the liver enzyme to catalyze the reverse reaction under physiological conditions. As indicated in Section VII,B, irreversibility of the total cycle is also maintained as a consequence of positive free energy change associated with reversal of Reaction 71.

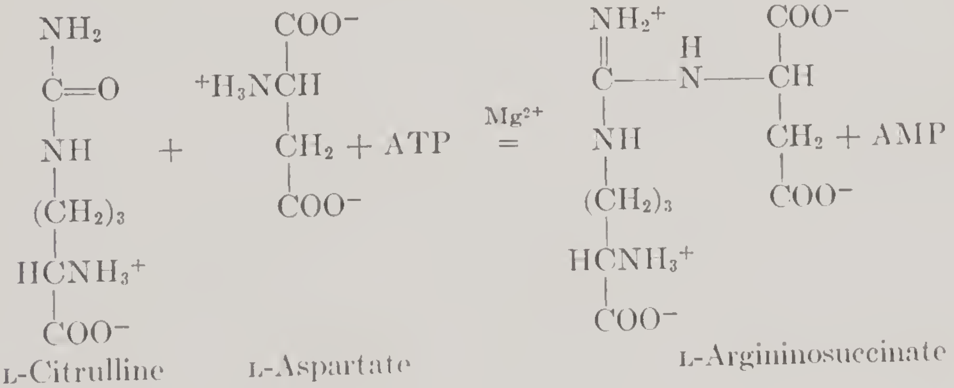
Escherichia coli exhibits a suppression of ornithine transcarbamylase activity when grown in the presence of arginine. This phenomenon is not attributed to inhibition of the enzyme itself, but rather to a blockage of enzyme synthesis (*321e*). Preformed enzyme-forming sites appear to be held under arginine suppression, and once this suppression is released by removing arginine from the medium, synthesis of ornithine transcarbamylase can proceed (*321f*).

There seems to be but little question that ornithine transcarbamylase occurs in lower and higher plants. In addition to the evidence cited above, the enzyme was demonstrated in an homogenate of a common mushroom (*321g*), and it is reported to occur in a mash prepared from the leaves of young (12–20 days) bean plants and in an acetone powder of *Canavalia ensiformis* (*321h*). Activity for ornithine transcarbamylase has also been demonstrated in cell-free extracts (sand-ground) of pure cultures of *Nostoc muscorum* (*321g*); this appears to be the first enzyme ever to be assayed successfully in a cell-free extract of a blue-green alga.

The equilibrium of Reaction 64 has been studied at pH 5.5 with the ornithine transcarbamylase of a pleuropneumonia-like organism (*321i*). The value obtained for the equilibrium constant (how formulated?) suggests that the reaction, under physiological concentrations of reactants and products, is thermodynamically feasible.

3. *Argininosuccinate Synthetase (Condensing Enzyme)*

Synthesis of arginine in liver is accomplished by the condensation of citrulline with aspartic acid to yield argininosuccinate; the latter intermediate is cleaved to form arginine and fumarate. The condensing enzyme, or argininosuccinate synthetase, catalyzes the reversible, ATP-dependent reaction (*237, 312, 322*) shown (Eq. 68).



(68)

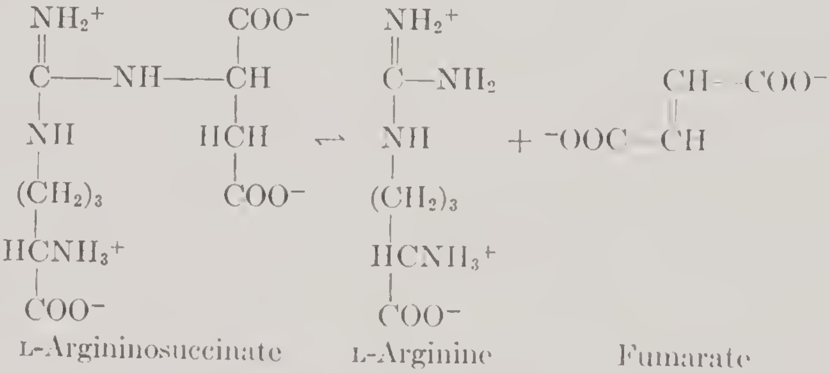
The enzyme has been partially purified from mammalian liver and kidney (*309, 323*) and is present in amphibian liver (*324*). Its occurrence must be presumed wherever the urea cycle is found unless evidence to the contrary is presented. The enzyme is found in yeast (*309, 322, 323*) and presumably in other microorganisms.

4. *Arginine Synthetase (Cleavage Enzyme)*

The enzyme which catalyzes the cleavage of argininosuccinate to arginine plus fumarate is widely distributed in nature. It occurs in mammalian kidney and liver (*322, 323*), and in adult anuran liver

(*324*), yeast (*322*), jack beans (*325*), pea seeds (*326*), *Chlorella* (*325, 327*) and *E. coli* (*322*), and in *Neurospora* (*328*).

This enzyme, as well as the condensing enzyme, has been studied extensively by Ratner and her co-workers (*322*) who have shown the products of the reaction to be arginine and fumarate. The reaction may be formulated as shown (Eq. 69).



L-Argininosuccinate L-Arginine Fumarate

$$\mathrm{{}^-OOC{-}CH(NH_3^+){-}(CH_2)_3{-}NH{-}C({=}NH_2^+){-}NH{-}CH(COO^-){-}HCH{-}COO^-} \rightleftharpoons \mathrm{{}^-OOC{-}CH(NH_3^+){-}(CH_2)_3{-}NH{-}C({=}NH_2^+){-}NH_2} + \mathrm{{}^-OOC{-}CH{=}CH{-}COO^-} \qquad (69)$$

The enzyme catalyzes the nonhydrolytic, nonoxidative cleavage of a C—N bond, and in this respect it is similar in action to aspartase which yields ammonia and fumaric acid; aspartate does not serve as a substrate, however. The reaction is freely reversible.

The enzyme system described by Reactions 68 and 69 affords the reversible synthesis of arginine from citrulline and aspartic acid in numerous and diverse plants, animals, and bacteria. This indicates that such a system is concerned with processes not necessarily connected with the specific function of disposing of ammonia via the urea cycle, e.g., synthesis of arginine from ornithine. Thus results consistent with the following proposed pathway have been obtained with *Escherichia coli* (*315, 329*): glutamate → *N*-acetyl glutamate → *N*-acetylglutamate-γ-semialdehyde → N^{α}-acetylornithine → ornithine → citrulline → arginine. [It is of interest in this connection, that *N*-acetyl glutamate is active as a cofactor for carbamyl phosphate synthetase (cf. Reaction 60) carried out by animals, whereas no such cofactor is required in the same reaction catalyzed by bacterial enzyme preparations (cf. Reaction 61).]

Sequential reversal of Reactions 69 and 68 is not to be confused with the reaction catalyzed by bacterial arginine desiminase (*330*) (cf. Reaction 21). The latter represents a hydrolytic cleavage of arginine.

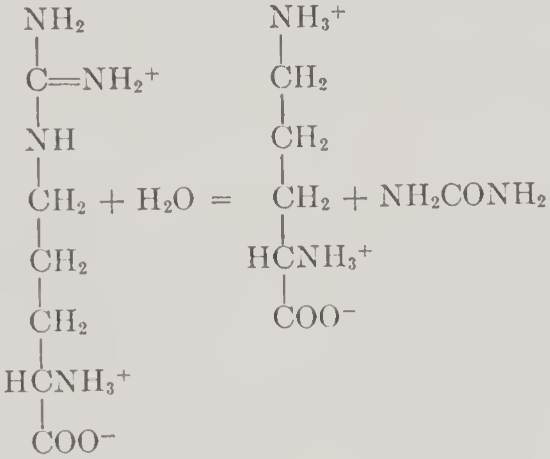
Synthesis of arginine from citrulline and ammonia in the presence of pyridoxal phosphate by a crystalline protein obtained from crayfish muscle has been reported (*331, 332*). The free energy change associated with Reaction 70

$$\text{Citrulline}^{\pm} + NH_4^+ = \text{Arginine}^{\overset{\pm}{+}} + H_2O \qquad (70)$$

is $\Delta F^{\circ\prime} = +6$ kcal. (estimated). Unless a substantially lower free energy change obtains it seems unlikely that such a reaction will occur physiologically unless it is coupled with an exergonic reaction. It is conceivable, on the basis of the free energy change estimated above, that in the presence of sufficient arginase, arginine could be held at a low enough concentration to provide a favorable ΔF. Canavanine (see Reaction 22) occurs in high concentration in the jack bean (*333*), and undergoes condensation with fumaric acid by the reverse of Reaction 69 to yield the argininosuccinate analog, canavaninosuccinate (*334*).

5. *Arginase*

Arginase catalyzes the reaction (*335–338*) shown (Eq. 71).



$$H_2N{-}C({=}NH_2^+){-}NH{-}CH_2{-}CH_2{-}CH_2{-}HCNH_3^+{-}COO^- + H_2O = H_3N^+{-}CH_2{-}CH_2{-}CH_2{-}HCNH_3^+{-}COO^- + NH_2CONH_2 \qquad (71)$$

L-Arginine L-Ornithine Urea

Mammalian liver seems to be the best source of the enzyme (*338, 339*). The enzyme is highly specific, attacking only the L-form of the amino acid and a few α-*N*-substituted compounds (*337–338*). Canavanine is also hydrolytically cleaved (*340*). Further discussion of properties of the purified enzyme appear elsewhere (*341–344*).

According to the Rule of Clementi (*345*), arginase occurs in the livers of animals which excrete urea, but not in those which excrete uric acid as their protein-N breakdown product. The arginase of birds (which excrete uric acid) is found in the kidneys. The hepatopancreas of snails is especially rich in arginase (*346*), but apparently not all gastropods contain the enzyme. Studies on the occurrence of the urea cycle in these organisms are lacking. Arginase is present in green plants and molds. The enzyme is present in most organs of the shark (*347, 348*). Adult amphibians, mammals, and some chelonian reptiles (turtles) excrete urea and possess arginase in their liver. Squamatine reptiles such as the lizard and snake excrete their waste nitrogen as uric acid and have been thought to lack arginase (*345, 349*). However, more recent studies (see Section VII,C,1,*c*) have shown the presence of arginase in lizard liver. The enzyme has been reported to occur in whale liver (*350*), fetal liver, kidney, and placenta (*351*), and in the liver and kidney of the scaly anteater (*352*) (*Manis* sp.).

Arginase levels in animals vary according to age (*353*) and to certain hormonal changes (*354–356*). Liver arginase increases during amphibian metamorphosis (*324, 357, 358*).

B. Energetics of the Biosynthesis of Urea

1. *Over-all Considerations*

The standard free energy of formation, $\Delta F_f{}^\circ$, for the synthesis of aqueous urea from the elements is —48.7 kcal. (Rossini, see reference *1*). From this value and that for $NH_4{}^+$ and CO_2 (gaseous), the free energy change for the following reaction, under conditions approximating those in the cell, has been calculated (*86*):

$$2\ NH_4{}^+\ (0.01\ M) + CO_2\ (0.05\ \text{atm.}) = \text{Urea}\ (0.01\ M) + 2\ H^+\ (\text{pH}\ 7) + H_2O \qquad (72)$$
$$\Delta F_{298}{}^\circ = +12.1\ \text{kcal.}$$

This is the minimum free energy a cell must expend for the synthesis of 1 mole of urea from ammonia and carbon dioxide under the specified conditions, regardless of the route of synthesis.

The synthesis of urea at pH 7 may be formulated by the over-all *chemical* reaction:

$$NH_4{}^+ + HCO_3{}^- + \text{Aspartate}^{\overset{-}{\pm}} = 2\ H_2O + \text{Urea} + \text{Fumarate}^{2-} + H^+ \qquad (73)$$
$$\Delta F_{298}{}^{\circ\prime} = +10.04\ \text{kcal.}$$

Reaction 73 was derived from the component reactions (see Table III) of the urea cycle as deduced from enzymatic experiments. Synthesis of urea as described by this reaction is associated with a positive change in free energy and hence is endergonic. Energy to drive the reaction is provided at various steps in the cycle by coupling with ATP breakdown, and the synthesis of urea proceeds exergonically. The energy-coupling reactions are omitted at this point so that the individual reactions may be examined and thus permit an approximation of the magnitude of the free energy changes associated with them. It is then possible to determine, *independently*, which reactions of the cycle proceed endergonically and are likely to require coupling with an exergonic reaction (ATP breakdown) so that synthesis of urea is favored.

The present section is devoted to the thermodynamics of the individual reactions. As far as the authors are aware, this is the first attempt to give a complete (albeit approximate) thermodynamic treatment of the individual steps of the urea cycle.

2. *Calculations*

Basic data are lacking for straight-forward calculation of the free energy changes associated with all of the individual steps of the urea cycle. However, some first approximations may be made with reasonable assumptions; these yield values consistent with what is known of the enzymology of the various steps.

Of the five purely chemical reactions given in Table III, the equilibrium constant of only one, the cleavage of argininosuccinate, has been determined. This yields $\Delta F_D°$ immediately, and from the $\Delta F_f°$ of fumarate^{2-} and of arginine$^{1-,2+}$ (estimated), the $\Delta F_f°$ of argininosuccinate$^{3-,2+}$ may be obtained. The $\Delta F_f°$ of ornithine$^{1-,2+}$ was approximated from the $\Delta F_f°$ of alanine$^{1-,1+}$ and from changes in $\Delta F_f°$ resulting from the introduction of two $—CH_2—$ groups within the chain and an $^+H_3N—$ group at the distal end. Sufficient data is thus obtained to calculate $\Delta F_E°'$ for the arginase reaction (Reaction E).

TABLE III

THERMODYNAMICS OF UREA SYNTHESIS[a]

Reaction	$\Delta F°'$ (kcal.)
A. $NH_4^+ + HCO_3^- = NH_2COOH + H_2O$ (−19.0) (−140.49) (−97.04)* (−56.7)	5.75
B. NH_2COOH + Ornithine$^{1-,2+}$ = Citrulline$^{\pm}$ + H_2O + H^+ (−97.04)* (−95.45)* (−118.96)* (−56.7) (−9.93)	6.90[b]
C. Citrulline$^{\pm}$ + Aspartate$^{2-,1+}$ = Argininosuccinate$^{3-,2+}$ + H_2O (−118.96)* (−166.99) (−222.35)* (−56.7)	6.90[b]
D. Argininosuccinate$^{3-,2+}$ = Arginine$^{1-,2+}$ + Fumarate^{2-} (−222.35)* (−75.17)* (−144.41)	2.77
E. Arginine$^{1-,2+}$ + H_2O = Urea + Ornithine$^{1-,2+}$ (−75.17)* (−56.7) (−48.7) (−95.45)*	−12.28
	Sum: +10.04kcal.

Sum: NH_4^+ + HCO_3^- + Aspartate$^{2-,1+}$ =
(−19.0) (−140.49) (−166.99)
$2H_2O$ + Urea + Fumarate^{2-} + H^+ ;
(−113.4) (−48.7) (−144.41) (−9.93);
$\Delta F°' = 10.04$ kcal.

[a] Figures in parentheses followed by asterisks equal $\Delta F_f°$ (estimated).

[b] $\Delta F_b°' + \Delta F_c°' = 13.80$. Assumption, $\Delta F_b°' = \Delta F_c°' = +6.90$ kcal.

The free energy of formation of carbamic acid was obtained from the assumption that substitution of an $—NH_2$ group for an $—OH$ group in carbonic acid yielded an amide with the same standard free energy of hydrolysis as asparagine. Another value was obtained from the assumption that the hydrolysis of urea to yield carbamic acid proceeded with the same standard free energy of hydrolysis as that of asparagine. The two values agreed to within about 3%; the average value was employed. The $\Delta F^{\circ\prime}$ of Reaction A is thus approximated.

The value of $\Delta F_{\Sigma}^{\circ\prime}$ is readily obtained from literature values of ΔF_f° for reactants and products. In Table III only the sum of the free energy changes associated with Reactions B and C could be obtained. Thus,

$$\Delta F_B^{\circ\prime} + \Delta F_C^{\circ\prime} = \Delta F_{\Sigma}^{\circ\prime} - (\Delta F_A^{\circ\prime} + \Delta F_D^{\circ\prime} + \Delta F_E^{\circ\prime}) = +13.8 \text{ kcal.} \quad (74)$$

If the assumption is made that the free energy changes of these two reactions are identical, ΔF_f° of citrulline$^{\pm}$ assumes a value of —118.96 kcal. Estimates of this value by several methods ranged from —124 to —130 kcal. We are in no position to evaluate these estimations. Until further thermodynamic data are available, or until more competent approximations can be made, values of $\Delta F^{\circ\prime}$ for the individual reactions (except D) are to be considered only as approximations. Decimal places in estimated values have been included only for purposes of formal calculation.

3. *Discussion*

The data obtained for the purely chemical reactions are summarized in Table III. Inspection of the free energy change associated with each reaction reveals pertinent information regarding points at which ATP might be expected to participate. Of the five reactions, only the hydrolytic cleavage of arginine by arginase proceeds with a negative change in free energy. The other reactions are endergonic.

ATP participates in reactions catalyzed by carbamyl phosphate synthetase and argininosuccinate synthetase (see Reactions 60 and 68). According to the most recent determinations, older values for the free energy of hydrolysis of ATP require revision. The value (359) selected here is —7.73 kcal. at pH 7:

$$\begin{gathered} \Sigma\text{ATP} + \text{H}_2\text{O} = \Sigma\text{ADP} + \Sigma\text{P}_i \\ \Delta F_{\Sigma}^{\circ\prime} = -7.73 \text{ kcal.} \end{gathered} \quad (75)$$

(where all ionic species at this pH are considered).[*]

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[*] Upon the cleavage of ATP at pH 7 a new ionizable hydrogen of phosphate appears. Since the pK_a of this group is about 7.2 it is not completely ionized. For convenience, the hydrogen ions associated with this group do not appear in this and subsequent equations, and they can be considered to be included with the symbols for the nucleotides. However, such hydrogen ions have been taken into consideration in calculating the free energy change.

Considered thermodynamically, synthesis of urea by Reaction 73 could be effected with 2 moles of ATP. In animal preparations however, 2 moles of ATP are required for the synthesis of 1 mole of carbamyl phosphate; another is used for the synthesis of argininosuccinate. In bacterial preparations only 1 mole of ATP is required for the synthesis of carbamyl phosphate. All attempts to separate the animal carbamyl phosphate synthetase into 2 enzymes have been unsuccessful. That prepared from bullfrog, *Rana catesbeiana*, has been obtained in a high state of purity with no indication that it consists of 2 distinct enzymes.

The carbamate kinase from bacteria, unlike the animal enzyme, does not require an added acyl derivative of L-glutamic acid. A difference in the mechanism of action for the synthesis of carbamyl phosphate by the two enzymes may thus be inferred. One can speculate that the formation of carbamyl phosphate can proceed either from ammonium and bicarbonate ions or from carbamic acid which is in equilibrium with these ions. A condition of low ammonium ion concentration is required in tissues of ureotelic vertebrates and the economy of the organism would best be served by effecting a synthesis of *bound* carbamic acid, for the equilibrium of carbamic acid in aqueous solution is predominantly in the direction of dissociation. Note that according to Reaction A in Table III, synthesis of carbamic acid is endergonic and has a calculated value of +5.75 kcal. This reaction could be driven nicely if coupled to breakdown of ATP. One mole of the nucleotide should suffice to maintain a concentration of carbamic acid (bound to the enzyme) sufficient to insure an appreciable rate of formation of carbamyl phosphate. The enzyme-bound carbamic acid could then be phosphorylated at the expense of another mole of ATP. On the other hand, bacteria are not faced with the problem of maintaining a low concentration of ammonia. Thus bacteria may synthesize carbamyl phosphate not from ammonium and bicarbonate ions but from carbamic acid which is in equilibrium with these ions. Only 1 mole of ATP would be required.

Details of the mechanism of action of the carbamyl phosphate synthetase are as yet obscure. As a working hypothesis in connection with the animal enzyme, the primary reaction may be regarded as the fixation of CO_2 as a high-energy derivative involving the participation of acetyl glutamate and ATP:

$$\text{E—Acetyl glutamate} + CO_2 + \text{ATP} \rightarrow [\text{E—Acetyl glutamate—}CO_2{\sim}P] + \text{ADP} \quad (76)$$

The postulated sequence leading to carbamyl phosphate is shown in Fig. 5.

Although both the animal and bacterial enzymes yield carbamyl phosphate as the main end product, it must be emphasized that their catalytic roles are fundamentally different. It would seem that ureotelic organisms have modified the primordial carbamyl phosphate synthetase present in certain bacteria. The bacterial enzyme probably functions chiefly as a means of generating ATP from carbamyl phosphate derived from citrulline and inorganic phosphate. The role of the enzyme in the synthesis of arginine and pyrimidines should not be discounted, however. Ureotelic forms may have appropriated this enzyme and with suitable embellishments have equipped it to serve in a sequence of reactions for disposal of ammonia. Inasmuch as carbamyl phosphate appears to be generated from citrulline by animal tissues only to a limited extent (*303*), carbamyl phosphate synthetase cannot be excluded from consideration as a component of the pathway for the synthesis of pyrimidines (see Section VII,E and Fig. 5) from ammonia and carbon dioxide.

TABLE IV

ENERGETICS OF THE ENZYMATIC STEPS IN UREA BIOSYNTHESIS[a]

<table>
<tr><th>Enzyme</th><th>Reaction</th><th></th><th>$\Delta F^{\circ\prime}$ (kcal.)</th></tr>
<tr><td rowspan="2">Carbamyl phosphate synthetase</td><td>$NH_4^+ + HCO_3^- + ATP =$ $H_2NCOOH + ADP + P_i$</td><td></td><td>-1.98</td></tr>
<tr><td>$H_2NCOOH + ATP =$ $H_2NCO{\sim}P + ADP$ 0?</td><td rowspan="2">-0.83</td><td rowspan="3">-1.66</td></tr>
<tr><td>Ornithine transcarbamylase</td><td>$H_2NCO{\sim}P$ + Ornithine = Citrulline + $P_i + H^+$ − 0.83?</td></tr>
<tr><td>ASA synthetase</td><td>Citrulline + Aspartate + ATP = Argininosuccinate + AMP + PP_i − 0.83</td><td></td></tr>
<tr><td>Arginine synthetase</td><td>Argininosuccinate = Arginine + Fumarate</td><td></td><td>$+2.77$</td></tr>
<tr><td>Arginase</td><td>Arginine + H_2O = Urea + Ornithine</td><td></td><td>-12.28</td></tr>
</table>

Sum: $NH_4^+ + HCO_3^- + H_2O$ + Aspartate$^{2-,1+}$ + 3ATP = Urea + Fumarate^{2-} + H^+ + 2ADP + AMP + PP_i + $2P_i$, $\Delta F^{\circ\prime} = -13.15$ kcal.

[a] Ionic species as in Table III.

Reactions B and C shown in Table III proceed with an over-all $\Delta F^{\circ\prime}$ of + 13.8 kcal. Two moles of ATP would suffice to drive both reactions: (*a*) conversion of carbamic acid (presumably enzyme-bound) to carbamyl phosphate at the expense of one ATP; (*b*) condensation of carbamyl phosphate with ornithine to yield citrulline, and coupling of the citrulline-aspartate condensation with one ATP to form argininosuccinate.

Although the cleavage of argininosuccinate by Reaction D, shown in Table III, proceeds with a slight positive $\Delta F^{\circ\prime}$, a rapid removal of the products would tend to overcome the slightly endergonic nature of the reaction. Fumarase is quite active in liver, and conversion to malic acid with an equilibrium ratio of malate/fumarate = 4 favors removal of fumarate. Under conditions obtaining in liver, the high activity of arginase would also aid to offset this positive free energy change, i.e., the actual ΔF may be less positive than $\Delta F^{\circ\prime}$.

If we assume the value of —7.73 kcal. for the $\Delta F^{\circ\prime}$ of the hydrolysis of ATP to either ADP or AMP, the $\Delta F^{\circ\prime}$ values for the *enzymatic* reactions may now be approximated. These reactions are summarized in Table IV. The various ionic species of the nucleotides and inorganic phosphate and pyrophosphate are considered and hence these are written in the uncharged form. The final over-all reaction* for the enzyme-catalyzed synthesis of urea may now be formulated:

$$\begin{aligned}\text{NH}_4^+ + \text{HCO}_3^- + \text{H}_2\text{O} + \text{Aspartate}^{\overset{-}{\pm}} + 3\ \text{ATP} = \\ \text{Urea} + \text{Fumarate}^{2-} + \text{H}^+ + 2\ \text{ADP} + \text{AMP} + \text{PP}_i + 2\ \text{P}_i \quad (77) \\ \Delta F^{\circ\prime} = -13\ \text{kcal.}\end{aligned}$$

The synthesis of urea proceeds enzymatically with a free energy change of —13 kcal. As indicated in Table IV most of this decrease in free energy takes place at the arginase step. The values for the free energy changes associated with the enzymatic reactions given in Table IV are in accord with instances in which free reversibility has been demonstrated. Generation of 1 mole of ATP from citrulline and inorganic phosphate in bacterial systems would proceed with a $\Delta F^{\circ\prime}$ of approximately 0.8 kcal. Since carbamic acid is unstable toward its dissociated form, a free energy change of ~ —5 kcal. should be obtained in the citrulline phosphorylase reaction (Reaction 64). Failure to demonstrate significant reversal of Reaction 66 catalyzed by ornithine transcarbamylase from liver seems to be an enzymatic, not a thermodynamic, impasse.

The cleavage of argininosuccinate to arginine and fumarate may be a rate-limiting step in urea biosynthesis in liver extracts (*322*). This reaction has associated with it a slight thermodynamic barrier of 2.77 kcal.

As far as is known, the highly exergonic hydrolysis of arginine by arginase cannot be coupled with generation of high-energy phosphate.

Again, it is pointed out that the $\Delta F^{\circ\prime}$ values reported here are in serious need of refinement. Determination of the standard free energies of formation of ornithine and citrulline would be helpful in this regard. The value taken for arginine is probably considerably in error.

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* For the sake of simplicity, hydrogen ions associated with the cleavage of ATP are not depicted as such in this equation. However, in the calculation of the free energy change *such* ions have been taken into consideration.

It must be emphasized that the free energy change for a reaction under physiological conditions is not necessarily that given by $\Delta F^{\circ\prime}$ (see Section II, and Vol. I, Chapter 2 and Vol. II, Chapter 1); the actual value may be about the same, much more negative or much more positive depending upon steady state thermodynamic concentrations of reactants and products at the site of reaction in the cell as well as the hydrogen ion concentration. Further refinements such as substituting thermodynamic activity for concentration and considering the temperature at which the reaction is catalyzed can also be made.

For the generalized equation,

$$aA + bB + cC + \ldots = \ldots + xX + yY + zZ \tag{78}$$

the actual free energy change, ΔF, is given by

$$\Delta F = \Delta F^{\circ} + RT \ln \left[\frac{\ldots (X)^x (Y)^y (Z)^z}{(A)^a (B)^b (C)^c \ldots} \right] \tag{79}$$

C. PHYLOGENETIC SURVEY OF UREA CYCLE ENZYMES IN VERTEBRATES

1. *Levels of Enzymes*

Levels of urea cycle enzymes have been determined in the livers of a number of classes of vertebrates, and some of these data are summarized in Table V. Numerical data were obtained, for the most part, from determinations made on fresh liver extracts by a refined technique for quantitatively assaying all of the enzymes from a single, small sample of liver tissue (*324*). Prior to the development and application of this technique, little information was available on the distribution and levels of these enzymes in various species. Notable exceptions, however, were the early comparative studies of Clementi (*345*, *349*) and Hunter and Dauphinee (*347*, *348*) on the distribution of arginase. The studies of these earlier investigators have served as a point of departure for subsequent investigations. The data obtained for the levels of arginase in these early studies gave an indication of only the approximate relative levels in various species as the result of carrying out assays under suboptimal conditions. An attempt to correct values so obtained to bring them in line with more recent data requires too many assumptions to yield anything but approximations; hence, only the presence (+'s) or absence (BLD = below the limit of detection) of arginase in such experiments are indicated in Table V. In those few instances where corrected older values can be compared with more recent ones, the two compare favorably in magnitude. Some other values are also reported in terms of +'s, for the original data yielded quantitative data of another type, namely units per mg. protein. A reinvestigation of arginase levels

TABLE V

LEVELS OF THE UREA CYCLE ENZYMES IN THE SUBPHYLUM VERTEBRATA (LIVER)

Classification	Common name	Units of enzyme activity per gram liver wet weight[a]				
		C.P.S.	O.T.C.	Cond. Enz.	Cleav. Enz.	Arg'ase
CLASS CHONDRICHTHYES						
SUBCLASS ELASMOBRANCHII						
Order Selachii						
Squalis sucklii (*347*, *348*, *360*, *361*)	Spiny dogfish	+?	+++			+++
Mustelus californicus (*361*)	Dogfish		41	6 ± 1	+	+
Order Batoidea						
Rhinobatus productus (*361*)	Guitarfish		36 ± 1	6.5 ± 1.5	+	+
Gymnura marmorata (*361*)	California butterfly ray		35	6 ± 1	+	+
Urobatus halleri (*361*)	Round sting ray			8 ± 2	+	+
Holorhinus californicus (*361*)	Bat sting ray		45			
Raia clavata (*349*)	Thornback skate					+
Torpedo ocellata (*349*)	Torpedo					+
CLASS OSTEICHTHYES						
SUBCLASS ACTINOPTERYGII						
Superorder Holostei						
Lepisosteus osseus oxyurus (*364*)	Garpike	BLD	BLD			
Amia calva (*364*)	Bowfin (mudfish)	BLD	BLD			
Superorder Teleostei						
Perca flavescens (*364*)	Yellow perch	BLD	BLD			
Cyprinus carpio (*364*)	Carp	BLD	BLD			
Salmo trutta fario (*364*)	Brown trout	BLD	BLD			
	Various teleosts (*349*)					+ (variable)
CLASS AMPHIBIA						
SUBCLASS LEPOSPONDYLI						

Order Urodela						
Suborder Proteida						
Necturus (369)	Mudpuppy	16 ± 4	2100 ± 1100	9.1 ± 1.0	56	20,000 ± 13,000
Suborder Salamandroidea						
Amphiuma means (369)	Congo eel	206 ± 36	10,000 ± 2,500	30	370	19,000
Suborder Ambystomoidea						
Ambystoma tigrinum (369)	Tiger salamander	360 ± 80	+ +			
Not identified (369)	Newt	378			> 121	
SUBCLASS APSIDOSPONDYLII						
Order Anura						
Bufo fowleri (369)	Fowler's toad	770				
Bufo marinus (369)	Giant toad	800 ± 83	17,000 ± 2,000	33 ± 6		23,000 ± 4,000
Bufo americanus (369)	Northern toad	780 ± 56				
Bufo terrestris (358)	(Tadpole stages)					+
Rana clamitans (364)	Green frog	+ + +				
Rana pipiens (369)	Leopard frog	+ + +		34		
Rana catesbeiana (369, 373)	Bullfrog	980 ± 120	9,700 ± 2,300	46 ± 10	498	25,000
Rana temporaria (349, 357)	Woodfrog					+ + +
Rana esculenta (349)	Water frog					+ + +
Rana heckscheri (358)	(Tadpole stages)					+
CLASS REPTILIA						
SUBCLASS ANAPSIDA						
Order Chelonia						
Chrysemys picta (369)	Painted turtle	640	13,000	45		6,000
Emys europa (349)	European pond turtle					+ +
SUBCLASS LEPIDOSAURIA						
Order Squamata						
Crotalis horridus horridus (369)	Timber rattlesnake	BLD		BLD	BLD	
Thamnophis sp. (369)	Garter snake					56–100

TABLE V (*Continued*)

Classification	Common name	Units of enzyme activity per gram liver wet weight[a]				
		C.P.S.	O.T.C.	Cond. Enz.	Cleav. Enz.	Arg'ase
Elgaria multicarinatus (*369*)	West. alligator lizard	BLD	BLD	BLD		5–30
Lacerta muralis (*349*)	Wall lizard					BLD
Anguis fragilis (*349*)	Slow worm					BLD
Coronella austriaca (*349*)	Smooth snake					BLD
CLASS AVES						
SUBCLASS NEORNITHES						
Superorder Neognathae						
Columba livia (*369*)	Pigeon			BLD	2?	BLD
Passer domesticus (*369*)	English sparrow		BLD			
Gallus domesticus (*349*)	Chicken					BLD
Turtur turtur, etc. (*349*)	Dove, various birds					BLD
Gallus domesticus (*380*)	Bantam fowl					+?
Gallus domesticus (*321g*)	Cornish–White Rock x		BLD		2?	~200
CLASS MAMMALIA						
SUBCLASS THERIA						
Infraclass Metatheria						
Order Marsupialia						
Didelphis virginiana (*369*)	Opossum	440	8,600			
Infraclass Eutheria						
Order Lagomorpha						
Oryctolagus sp. (*369*)	Rabbit	213	6,000	26	180	13,000
Order Rodentia						
Suborder Myomorpha						
Rattus norvegicus (*369, 421*)	Rat	690 ± 150	23,100 ±900	75 ± 15	200	28,000 ±4,000
	Mouse tissue culture 1469 N.I.H. (*369*)	BLD	BLD	BLD	+	~1000
Suborder Sciuromorpha						
Not identified	Ground squirrel	++	+++			

Suborder Caviamorpha						
Cavia sp. (*364*)	Guinea pig	++	+++			
Erethizon dorsatum (*364*)	Porcupine	++	++			
Order Carnivora						
Infraorder Aeluroidea						
Felis domesticus (*364*)	Domestic cat	+++	+++			
Canis familiaris (*349, 425*)	Dog	++	+++			++
Infraorder Arctoidea						
Potos caudivolvulus (*369*)	Kinkajou	380	3,550	8–19	>264	18,700
Order Artiodactyla						
Suborder Suina						
Sus domesticus (*349, 369*)	Pig	++	+++	++	51	++
Suborder Ruminata						
Bos taurus (*95, 323, 369*)	Ox	>360	27,000	92	++	++
Capra hircus (*369*)	Goat	490	24,000	>70	++	8,800
Order Chiroptera						
Eptesicus fuscus (*364, 369*)	Big brown bat	2,100	25,000	>47	++	26,000
Myotis lucifugus (*369*)	Little brown bat	2,800	53,000	>90	+++	52,000
Order Primates						
Suborder Anthropoidea						
Superfamily Cercopithecoidea						
Macaca rhesus (*369*)	Rhesus monkey	420	14,000	41	++	11,000
Superfamily Hominoidea						
Homo sapiens (*369*)	Man	240 ± 17	8,500 ± 550	52 ± 4	174 ± 6	7,800

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[a] The abbreviations are: C.P.S. = carbamyl phosphate synthetase; O.T.C. = ornithine transcarbamylase; Cond. Enz. = condensing enzyme; Cleav. Enz. = cleavage enzyme; Arg'ase = arginase; + = present; BLD = below limit of detection by method of assay. Numerical values of activity are in terms of micromoles product/hour/gm. wet weight of liver. In cases where acetone powders were employed, values are corrected to wet weight by assuming 1 gm. acetone powder = 5 gm. wet weight liver. A variety of methods were employed: glycerol extracts (*347, 348*); extract of acetone powder (*95, 323, 361, 364, 425*); water (*349, 357, 358*); water extract of lyophilized liver (*R. hecksheri* (*358*); cetyltrimethylammonium bromide homogenate (*369, 373, 421*). The +'s give approximate relative levels and should be compared vertically.

throughout the vertebrate phylum is indicated and, until this data is available, a discussion of the level of this enzyme as it occurs in various species can be dealt with only in broad terms. Levels of enzymes reported numerically in Table V are expressed as micromoles of product formed per hour per gm. wet weight of liver ($T = 38°$).

a. Fish. The elasmobranchs — sharks, rays, and skates — seem to possess all of the urea cycle enzymes, although the occurrence of carbamyl phosphate synthetase (*360*) is not well documented. There can be no question as to the occurrence of arginase in the liver of elasmobranchs (*347, 348*). The presence of the argininosuccinate cleavage enzyme has not been demonstrated directly; its presence was inferred from the observation that urea was produced in the presence of an extract of liver acetone powder (*361*) (known to contain also arginase) and substrates for the condensing enzyme (see Eq. 68). As is the case with mammals and amphibians, shark liver ornithine transcarbamylase is found in the mitochondrial fraction of liver.

The level of arginase in the liver of the shark, *Squalis sucklii,* would appear to be about the same, possibly slightly less, than in mammalian liver, e.g., the rat. This fish shows high levels also in the heart and kidney, and this character may be common to all the elasmobranchs (*347, 348*).

Elasmobranchs maintain a high concentration of urea in the blood which serves to maintain them at near isotonicity with sea water (*362, 363*). The concentration may be as high as 2 to 2.5% compared with levels of about 0.01 to 0.03% in all other vertebrates. Maintenance of this high concentration is aided in part by the impermeability of the respiratory epithelium to this substance and its active reabsorption by the kidney tubules. The concentration in the relatively few freshwater species is less than in those of marine habitat. The freshwater sawfish (*Pristis microdon*) makes osmotic adjustments in moving from brackish water to fresh water by increasing its flow of urine with a consequent reduction of blood urea (*363*). Part of the success of elasmobranchs in invading the sea from a fresh water habitat may be attributed to their passive ability to make osmotic adjustments by increasing the concentration of blood urea.

The subclass Holocephali of class Chondrichthyes, containing the chimaeras, has not as yet been examined for the occurrence of any of the urea cycle enzymes except arginase. Low levels of arginase have been observed in both liver and kidney of the ratfish, *Hydrolagus colliei,* the kidney presumably possessing the most activity (*348*).

In contrast to the cartilaginous fish mentioned above are the bony fish (Class Osteichthyes) containing the highly advanced superorder

Teleostei, in which group the greatest number of fish belong. Arginase has been found to occur in the liver of every teleost examined (*347*), but at levels much less than those of the liver of elasmobranchs. Kidney possesses about one-tenth the activity of liver. Arginase is reported to occur in the hearts of the teleost families Clupeidae (herring), Salmonidae (Coho salmon) and Ebiotocidae (silver and blue perch), but not in Scorpaenidae (cods), Hexagrammidae (Ling cod), Cottidae (sculpin and bullhead) or Pleuronectidae (flatfish) (*347*).

Carbamyl phosphate synthetase and ornithine transcarbamylase activities could not be demonstrated in the livers of any teleosts or holosteans examined (*364*). This is not surprising since it has been known for some time that such fish excrete but little urea, their excretory nitrogen being accounted for by ammonia, trimethylamine oxide, and other identified and unidentified nitrogenous compounds (*365*). No information seems to be available for the chondrosteans (*Polypterus*, sturgeons, paddlefish).

The *bony* fish discussed above are all members of the subclass Actinopterygii (ray-finned fish). Another subclass of Osteichthyes is Choanichthyes, members of which possess internal nostrils and fleshy fins. This group contains the extant dipnoans or lungfish (only three genera) which are of particular phylogenetic interest because of their similarities to ancestral types. The dipnoans are also of particular interest in the present context because of the ability of two of the genera to aestivate and store up large quantities of urea (*366*). Levels of urea cycle enzymes have not yet been measured in these primitive fish, but it may be presumed that the complement of urea cycle enzymes is present, at least during aestivation. The subclass Choanichthyes also contains the Crossopterygii, paleozoic fossils thought to be broadly ancestral to the land vertebrates (*367, 368*).

Levels of enzymes in the jawless vertebrates (Class Agnatha) such as the hagfish and lampreys seem not to have been investigated. Because this group is thought to stem closely from the base of the vertebrate tree, it would be of considerable interest to determine if they possess any of the urea cycle enzymes. The thesis is developed in a later section that the primitive piscine ancestors of the higher vertebrates possessed urea cycle enzyme activities.

b. Amphibians. The urea cycle enzymes have been demonstrated to occur in the livers of all amphibians investigated (*369*) including the mudpuppy (*Necturus maculosus*), Congo "eel" (*Amphiuma means*), tiger salamander (*Ambystoma tigrinum*), newts, toads (*Bufo* spp.), and frogs (*Rana* spp.) The only major group for which data are lacking is that of the legless amphibians (Apoda=Gymnophiona=caecilians).

Of considerable interest is the observation that the levels of carbamyl phosphate synthetase in the livers of amphibians reflect the extent of ontogenic development (*369*). Thus if one considers that the present limit of amphibian development is reached in the tailless and lung-breathing frogs and toads, one finds a neat correlation with the extent of this development and the level of this enzyme. This is brought out in the histogram (Fig. 6) where it is seen that the level of the enzyme increases from a low level in the mudpuppy to a high level in the toad and frog. In this series, development is seen as a progressive loss of larval characteristics such as gills, spiracle, and tail. Each of the forms is sexually mature even though there may be a retention of larval characteristics possessed by a higher form at some time during the latter's own ontogeny. [The Mexican axolotl, similar in appearance and development to the mudpuppy, excretes roughly equal quantities of ammonia and urea (*370*). The axolotl is a neoteinic form of the tiger salamander of North America (*367*) and will metamorphose upon treatment with thyroid agents, whereas *Necturus* will not so respond (*371*).]

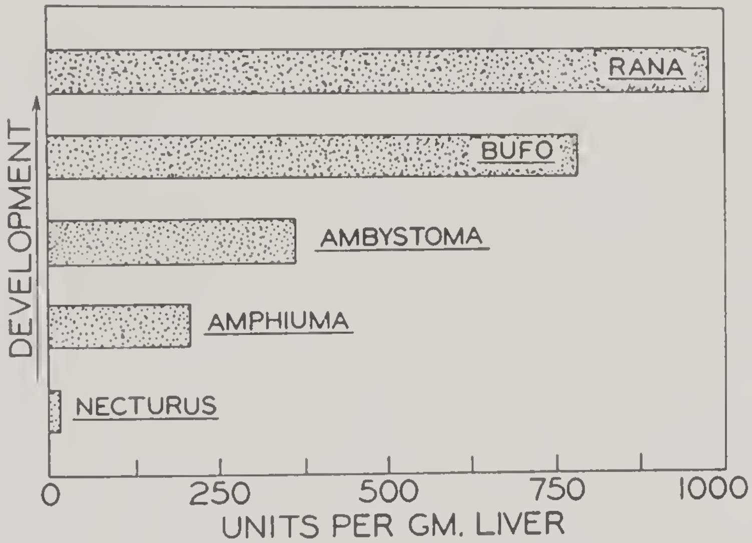

FIG. 6. Amphibian development vs. liver carbamyl phosphate synthetase.

A similar situation obtains for all of the enzymes during the ontogeny of the frog (*324, 372, 373*). It has been known for some time that tadpoles excrete their nitrogen mainly in the form of ammonia. During metamorphosis, ammonotelism is abandoned for ureotelism, and throughout the course of metamorphosis the level of the urea cycle enzymes in larval liver increases. The first observation of this phenomenon was made in the case of arginase (*357, 358*). One must look elsewhere, however, to explain the shift from ammonia to urea excretion, for arginase in larval, premetamorphic tadpoles is present in amounts in excess of that needed to support urea synthesis in postmetamorphic tadpoles or even in the adult frog. Conclusive evidence that there is some type of induction of the urea cycle at metamorphosis has been obtained by demonstrating the enhancement of all enzyme activities of the urea cycle during the course of metamorphosis (*372, 373*). It has also been found that the levels of all enzymes are sufficient to account for the over-all rate of urea synthesis as judged by the rate of urea excretion in intact tadpoles. This latter observation is especially important in view of the suggestions of alternative schemes of urea formation (see Section VII,D).

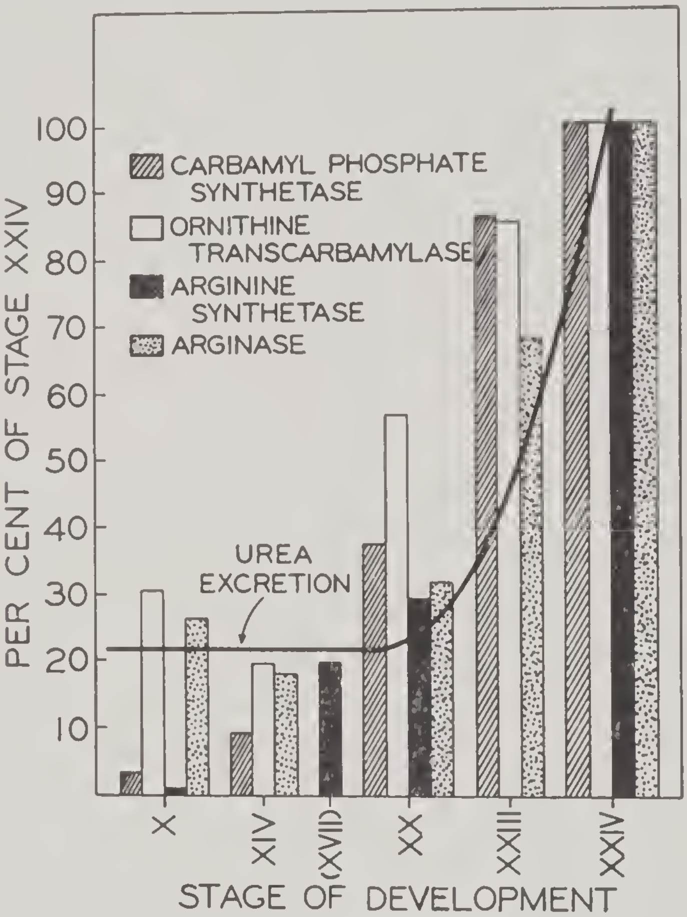

FIG. 7. Urea excretion and development of the enzymes of the urea cycle in the metamorphosing tadpole (*Rana catesbeiana*) (*373*).

Techniques developed in the authors' laboratory for the estimation of urea cycle enzymes (*372*) were first applied to a study of the level of the enzymes in developing tadpoles of the bullfrog, *Rana catesbeiana*. The course of development in the tadpoles was followed by examination of gross morphological changes to which stage numbers may be assigned (*374*). Another good approximation of the extent of development is given by the ratio of hind leg length to tail length.

The onset of urea production in these tadpoles is shown graphically in Fig. 7. A sharp increase in urea excretion with a concomitant decrease in ammonia excretion occurs at Stage XVIII, the latter corresponding to the onset of metamorphosis as defined elsewhere. An increase in activity of urea cycle enzymes occurs at the beginning of metamorphosis, precisely where a shift from ammonia to urea excretion is observed in intact tadpoles. Activities increase beyond this stage whether they are based on units per animal, units per gm. of liver, or units per mg. protein (specific activity). On the basis of micromoles of product produced by each enzyme/hour/gm. liver under assay conditions, the enzyme activities fall in the following order: arginase > ornithine transcarbamylase > carbamyl phosphate synthetase > arginine synthetase system. Assay for the latter system includes both the condensing and cleavage enzymes. Though the activity of the arginine synthetase system is quite low compared with the other enzymes, and thus limits the overall rate of urea synthesis, it is sufficient to account for urea production by the tadpoles at any given stage of development. Preliminary evidence indicates that in amphibians the citrulline-aspartate condensation step (Eq. 68) is rate-limiting (*372*). The order of decreasing activities listed above seems to hold in all ureotelic animals.

c. *Reptiles.* The squamatine reptiles—lizards and snakes—are known to be uricotelic (*365, 374a*), and their livers do not contain the full complement of urea cycle enzymes. Clementi (*345, 349*) could not demonstrate arginase activity in either liver or kidney of those reptiles. Recently, however, the authors (*369*) examined the liver of the western alligator lizard, *Elgaria multicarinatus*, and were able to demonstrate feeble arginase activity (5–30 micromoles urea per hour per gm. wet weight without preincubation with Mn^{2+}). The reaction showed an absolute dependence upon arginine, and a partial dependence upon manganese. Activity was lost upon boiling the extract and no urea production could be demonstrated when urease was included in the assay system. If this finding can be confirmed, Clementi's long-standing rule that arginase does not occur in the *liver* of uricotelic vertebrates would no longer apply. Inasmuch as arginase activity in birds is largely confined to kidney (see below), this organ of squamatine reptiles should be re-examined for the occurrence of arginase by techniques more sensitive than were employed in the early studies of Clementi.

The Crocodilia are considered as ammono-uricotelic (*375*),* and probably do not synthesize urea by the Krebs-Henseleit ornithine-urea cycle. No data seems to be available for the occurrence of enzymes of the cycle in this group.

Chelonian reptiles, represented by numerous species of aquatic, amphibious, and terrestrial turtles, are an extremely versatile group. In general, turtles excrete nitrogen in the form of ammonia, urea, and uric acid (*376–378a*), and the proportion of these three is related to habitat. In going from almost wholly aquatic to arid-dwelling species, one finds a progressive increase in the amount of uric acid and a decrease in the amount of ammonia excreted. Excretion of urea, on the other hand, is high in the semiaquatic and damp-terrain terrestrial species, while lower percentages of excreted nitrogen are accounted for as urea in species occupying both environmental extremes in relation to water supply. All of the urea cycle enzymes have been demonstrated in the liver of the painted turtle, *Chrysemys picta* (*369*), and arginase, at least, is known to occur in the liver of the semiaquatic *Emys europaea* (= *E. obicularis*) (*379*). *Testudo graeca*, a species living under almost desert conditions. and which excretes mostly uric acid, is nevertheless thought to possess all components of the urea cycle. It would seem that the Chelonia stand in contradistinction to the other reptiles in being able to effect the synthesis of urea *de novo* from ammonia and bicarbonate far above the ability of other reptiles to do so, if the latter can indeed synthesize urea at all.

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* For excretion by alligator embryo see Clark *et al.* (*375a*). It is not known whether the urea excreted by the embryo is derived by reactions of the urea cycle or merely by the action of arginase on arginine.

d. Birds. In his early studies, Clementi (*349*) concluded that arginase was absent from avian liver. For example, arginase assays gave negative results with *Gallus domesticus*, *Columbia livia*, *Turtur turtur*, *Fringuilla cloris* and *Coturnis communis*. There has not been universal acceptance of this idea, for Edlbacher as well as Chaudhuri (see *380*) have claimed that avian liver contains arginase. To throw some light upon this controversy, the authors carried out an arginase assay on pigeon liver under conditions where arginase activity could be demonstrated in lizard liver. If pigeon liver contains arginase at all, the level of the enzyme must be less than that needed to produce about 1 μmole of urea per hour per gm. wet weight of liver. However, arginase activity has now been demonstrated with chicken liver extracts (see Table V).

But while arginase is difficult or impossible to demonstrate in avian liver, avian kidney possesses considerable activity (*349*), possibly less than that found in typical ureotelic liver. The location of arginase in avian kidney instead of in liver may serve to minimize the breakdown of arginine. Arginine is an essential amino acid for birds (*381*). If liver contained appreciable arginase activity, absorption from the gut via the hepatic-portal system might subject the amino acid to hydrolytic cleavage, which would certainly not be advantageous to avian economy. The presence of arginase in kidney, on the other hand, serves to provide ornithine for detoxification of benzoic acid as ornithuric acid (*382*).

In 1931 Needham (*383*) published a graph on the nitrogen excretion products in developing chick embryos. This graph has appeared in at least four other textbooks with the implication that during embryonic development the bird passes through an evolutionary recapitulation—from ammonia to urea to uric acid excretion—with the resulting temporary acquisition of the urea cycle. But Needham and co-workers (*384*) long ago concluded (and more recently it has been shown, see *385*) that this is not the case at all. The formation of urea in chick embryos finds explanation in the action of arginase on arginine derived from yolk protein. Recently, the idea of such recapitulation was revived (*386*), but because adequate experimental details were lacking in the reference cited, no appraisal of the data may be attempted.

e. Mammals. All of the urea cycle enzymes have been found in mammalian liver wherever sought (*364, 369*). Arginase is present also in mammalian kidney and testes (*338*). The condensing and cleavage enzymes of kidney are quite active (*323*). The numerous species investigated are tabulated in Table V, and only a few comments need be made.

Of all the species investigated, the two bats, *Eptesicus fuscus* and *Myotis lucifugus,* possessed the highest levels of carbamyl phosphate synthetase.

It is instructive to compare the rate of urea synthesis in man (as judged by the rate of excretion) with the level observed for the arginine synthetase system [slowest step(s)] in cell-free extracts of liver. A 70 kg. human excretes about 20 gm. or about $\frac{1}{3}$ mole of urea per 24 hours. The level of the arginine synthetase system in two samples of human liver (see Table V) yielded an average value of 52 μmoles of product per hour per gm. wet weight of liver. The liver wet weight of a 70 kg. human is about 1500 gm.; the total liver is thus able to synthesize a maximum of $52 \times 1500 \times 24 = 1.87 \times 10^6$ μmoles or approximately 1.9 moles of urea per day. This rate of synthesis is 6 times the observed normal rate ($\sim$0.33 moles per day). It is thus seen that the rate-limiting reaction(s) of the urea cycle *in vivo* can operate at substantially lower rates than observed *in vitro* and still account for the rate of urea synthesized and excreted. Presumably, *in vivo*, the enzymes are not saturated with substrate, and thus the rate of the rate-limiting reaction becomes hyperbolically dependent upon substrate concentration. It thus follows that man can handle, as is known, certain loading doses of amino acids and ammonia.

2. *Urea Cycle Enzymes and Vertebrate Evolution*

The evolutionary importance of nitrogen excretion products has been recognized and discussed (*362, 363, 365, 383, 387, 388*). Excretion mechanisms and patterns of nitrogen excretion are treated elsewhere in this treatise (Vol. II, Chapters 8 and 9). In general, the nature of the nitrogen excretion product of protein degradation depends, among other things, upon the abundance of water (in either an absolute or osmotic sense) in an animal's embryonic environment. Thus, most aquatic animals are ammonotelic, while land forms are ureotelic or uricotelic. Ammonia is relatively toxic, and while aquatic animals have a large reservoir in which to excrete this substance, other animals have evolved apparently only as they were able to acquire accessory mechanisms for disposal of ammonia.

Birds and saurian reptiles convert ammonia to uric acid, a relatively innocuous substance which demands little water for its excretion. Amphibians, some chelonian reptiles, and mammals, on the other hand, excrete urea. Urea is a substance of low toxicity but one which demands relatively more water for excretion than does uric acid. In the case of marine and desert mammals, dehydration is circumvented through the use of metabolic water and the water content of foods, the action of efficient kidneys producing a highly concentrated urine, and/or the diminution of water loss by various physiological devices. Marine birds and reptiles, while requiring little water for excretion of the partially soluble uric acid, can however drink sea water and secrete a solution hypertonic to this via specialized salt glands (*389*).

Excretion of uric acid by insects (*374a*) appears to be an instance of evolutionary convergence with the uricotelic vertebrates.

Much evidence is available to suggest that life originated in the sea and penetrated to estuarine waters. Invasion of land was not accomplished cataclysmically; rather, some limicolous animals akin to our present-day amphibians left the water to spend longer and longer intervals on dry land. Some progeny of these, by various and diverse evolutionary mechanisms (*390*), became practically independent of an external aqueous environment. Frog larvae, i.e., tadpoles, mimic this evolutionary sequence, and do so in a highly suggestive manner. Young tadpoles excrete ammonia, and during metamorphosis ammonotelism is abandoned. By the time this amphibian has functional limbs it is prepared physiologically for invasion of land, and it now excretes urea.

The data summarized in Table V provide enzymatic evidence for the absence of functional urea cycle activity in squamatine reptiles and birds and for the presence of such activity in amphibians, mammals and chelonian reptiles. Such findings indicate that certain associated enzyme activities were lost, gained, or somehow modified during the course of vertebrate evolution. While no final answer as to the nature of these enzymatic changes can be offered, certain assumptions can be made which provide a simple and tentative explanation for the distribution of urea cycle enzymes in the livers of extant vertebrates. And it seems advisable to restate and amplify, in the light of more recent data, the sequence of evolution of nitrogen excretion mechanisms presented elsewhere.

The evolutionary tree, as modified from several sources (*363, 367, 368, 391*) from piscine ancestors to the higher vertebrates is shown in Fig. 8. The assignment of + or − in Fig. 8 indicates the presence or absence of the full complement of urea cycle enzymes in certain groups. Signs in parentheses indicate *postulated* presence or absence in certain groups; these are tentative and subject to revision as more evidence becomes available. The picture (admittedly speculative) which emerges is the following:

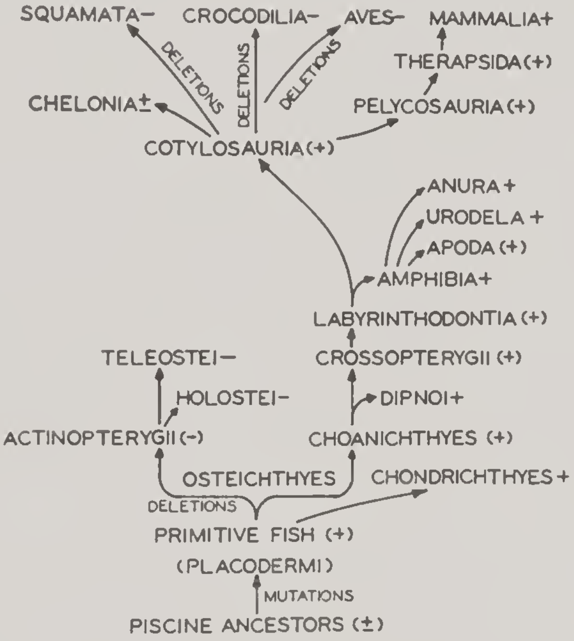


FIG. 8. Evolutionary tree of the vertebrates. See text for meaning of signs.

At some time there arose primitive fish, possibly the Placodermi, possessing the complement of urea cycle enzymes; these primitive fish were derived from ancestors which gradually acquired enzymes of the urea cycle by mutations. As depicted elsewhere (*373*), the urea cycle can operate as such in the intact animal presumably only with the cooperation of 4 ancillary reactions providing a source of aspartate for the synthesis of argininosuccinate, namely, fumarate → malate → oxalacetate → aspartate, and α-ketoglutarate → glutamate. Thus, *at least* 9 enzymes, and presumably *at least* 9 genes, were required before the urea cycle could function as a mechanism for the disposal of ammonia as urea; it is not logical to assume that these enzymes arose in one evolutionary step, but rather, that the evolution of the cycle was quantized.

The widespread occurrence of enzymes of the urea cycle in a variety of plant, animal, and bacterial sources indicates that these enzymes were being exploited in nature before final acquisition of the cycle as a mechanism for the excretion of nitrogen (Section VII,A). The final acquisition was likely a process involving a series of mutations serving the economy of primitive and diverse forms in various ways. Thus, the enzymes preceding arginase in the cycle offer a route for the synthesis of arginine for protein synthesis and for synthesis of the phosphagens, arginine and creatine phosphate. In addition, carbamyl phosphate synthetase provides a pathway for pyrimidine biosynthesis (see Section VII,E).

The assignment of an active urea cycle with its complement of enzymes to certain primitive fish is based upon the ureotelism exhibited by sharks which derived at about the same time from the same general stock as did the Actinopterygii and the Choanicthyes. It is suggested that certain deletions of enzymes of the urea cycle occurred in the evolvement of the Actinopterygii, for the modern representatives do not possess all of the enzymes of the urea cycle. Choanichthyes, the other major subclass of Osteichthyes which includes the Crossopterygii (Middle Devonian bony fishes), is assigned all of the urea cycle enzymes as are the Labyrinthodontia (primitive amphibians), since an early branch of this stem, the Dipnoi (lungfish) and a later branch, the Amphibia, are ureotelic.

The Cotylosauria ("stem reptiles") are also assigned an active urea cycle since an early branch, the Chelonia, possesses members containing the urea cycle enzymes. The reptilian Pelycosauria and Therapsida, antecedent to Mammalia, are derived from the main stem stock of reptiles and would be thought to still possess the urea cycle enzymes. Deletions within the stem reptiles are presumed to have occurred, for Aves, Crocodilia, and Squamata do not excrete appreciable urea, nor are they known to possess the complement of urea cycle enzymes. It is not at present clear whether such deletion of activity corresponds to a true genetic loss or merely to a suppression of activity through unknown mechanisms.

Emphasis on uric acid synthesis is presumed to have occurred sometime during early reptilian evolution, for chelonian reptiles have the ability (as indicated above) to excrete their nitrogen as ammonia, urea, and/or uric acid depending upon species and environment.

Alternative hypotheses can certainly be offered to explain these enzymatic alterations in the evolutionary tree. For example, there could have been deletions and back mutations (to regain the enzymes) or enhancement or suppression of activity of certain enzymes of the urea cycle almost anywhere in the scheme. The term "deletion" should at present be interpreted to mean the lowering of enzymatic activity to such a level that the urea cycle no longer functions to any significant degree as a system for the disposal of ammonia as urea. The assignment of urea cycle activity in extinct forms can of course come only from indirect evidence. Knowledge on the occurrence or absence of urea cycle enzymes in the livers of species occupying peculiar phylogenetic positions such as the platypus, coelacanths, and the New Zealand sphenodon, could provide valuable information.

In the course of development of the above ideas, attention was called to the prior suggestion of Smith (*363*) that Therapsids and Crossopterygians were ureotelic. The ideas of Smith were developed through a consideration of renal functions and mechanisms, and relied heavily, as do the present ideas, upon the findings of paleontology and comparative anatomy. Renal and biochemical excretion mechanisms are closely allied, and with increasing advances in our understanding of the basic biochemical mechanisms involved, a clearer picture of the evolutionary development of the vertebrate phylum is beginning to emerge.

D. ALTERNATIVE CYCLES FOR UREA BIOSYNTHESIS

While the urea cycle as outlined in the previous section has in general been accepted as the major if not the only pathway leading to arginine and urea synthesis, several reports have appeared in the literature which challenge the obligatory role of this cycle. In general the exceptions taken to the proposed enzymatic pathway relate to the role of citrulline and the possibly unique roles of glutamine and amino acids. Krebs (*392*) has discussed certain aspects of this problem.

Gornall and Hunter (*393*) early pointed out that the catalytic ratio, urea synthesized/citrulline concentration, in liver slices, was lower than the ratio realized with ornithine when the latter was at low concentration. This effect has been more recently studied by Bronk and Fisher (*394–396*). While Gornall and Hunter interpreted their findings as being entirely consistent with the operation of the ornithine cycle as proposed by Krebs and Henseleit, and stated that formulation of alternative schemes was superfluous, Bronk and Fisher interpreted their own findings as evidence for an alternative cycle. The proposal by Bronk and Fisher (*395*) that ornithine and citrulline may function in somewhat independent cycles, which both lead to urea synthesis, is based on the observations that citrulline is less effective catalytically in slices under certain conditions than is ornithine and that the two compounds are competitive under certain conditions. Experiments with cell-free preparations have established that the specific activity of C^{14}-citrulline synthesized and isolated is the same as that of the $C^{14}O_2$ fixed and the C^{14}-urea isolated (*397*). If citrulline were not an obligatory intermediate, one would expect a lower specific activity of the isolated citrulline. Hirs and Rittenberg (*398*), employing liver slices and isotopically labeled metabolites, established that citrulline behaved as a true intermediate in arginine synthesis from ornithine. It would thus appear that the position of citrulline as an obligatory intermediate in the biosynthesis of urea is experimentally established and that the position taken by Bronk and Fisher is a matter of interpretation of the conditions which obtain in slices.

The following possibilities, in addition to those of permeability effects, for explaining the observations of Gornall and Hunter, and Bronk and Fisher, might be considered. If free citrulline were not an intermediate in the cycle, but rather citrulline phosphate (or some similar derivative), the failure of citrulline to be as effective as ornithine under certain conditions would be explained. However, to date no evidence for this has been reported. Studies with a partially purified carbamyl phosphate-ornithine transcarbamylase failed to demonstrate the synthesis of such an intermediate (*95*). It must be recognized that arginine synthetase is one of the slowest of the enzymatic steps in the ornithine cycle. There will also exist in slices the problem as to whether the aspartic acid level is adequate under the conditions employed to insure maximum operation of this rate-limiting step. The possibility exists that carbamyl phosphate formed in the absence of added ornithine may deplete the aspartic acid concentration of the slice by forming carbamyl aspartic acid (cf. Section VII,E). In the presence of ornithine, carbamyl phosphate would not be as readily diverted because of the high activity of ornithine transcarbamylase. Whatever the basis for the apparent discrepancy in the catalytic efficiency of ornithine and citrulline, it is clear that the interpretation of data of the type obtained by Bronk and Fisher with slices will be difficult because of the complexity of the many factors (particularly permeability) which are involved. The proponents of other pathways for urea biosynthesis could make a substantial contribution if they would provide direct experimental evidence for the existence of additional enzymatic pathways.

A number of reports have appeared which ascribe to glutamine a special role in urea biosynthesis. Bach and Smith (*399*) have recently proposed that the α-amino and amide groups of glutamine may both be utilized for urea biosynthesis without previous conversion to ammonia. These authors have proposed that CO_2 may be fixed in the glutamine (or isoglutamine) molecule to form a ureido compound which is then cleaved to form urea. These conclusions with liver slices are based to a large extent on the fact that glutamine under certain conditions appears to be more effective than ammonia in urea biosynthesis. Here again the problem of permeability is difficult to evaluate. In sharp conflict with the proposal by Bach and Smith are the experiments of Hirs and Rittenberg (*398*) in which N^{15}-amide labeled glutamine was used and which revealed no specific or unique function of the amide nitrogen of glutamine in urea biosynthesis.

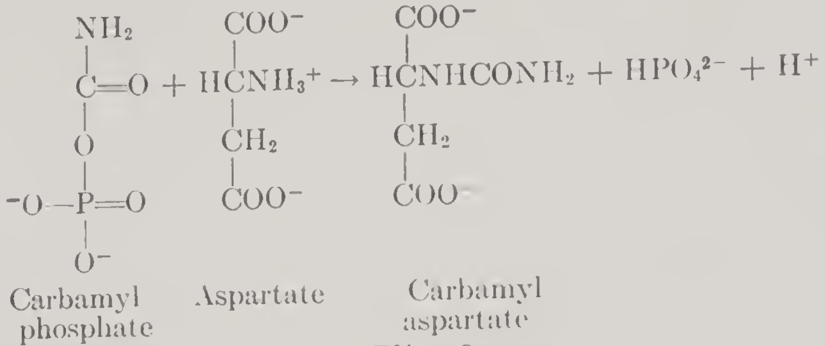
Cedrangolo and his co-workers (*400*) have published data on urea excretion in rats after oral administration of ammonium chloride and of L-amino acids. It was reported that feeding ammonium chloride has little effect on urea excretion while amino acids caused an increase. On this basis they claim that free ammonia is not involved in any step in urea biosynthesis but rather that the urea biosynthetic mechanism utilizes amino groups transferred from amino acids directly to intermediates of the urea cycle. These authors propose that carbamyl phosphate may in fact be synthesized in the animal from amino acids rather than from free ammonia. Experiments in which a highly active and highly purified carbamyl phosphate synthetase was used revealed that of a large number of amino acids and derivatives (including glutamine) tested in place of ammonia, none was active (*401*). Further, it has been observed that carbamyl phosphate synthetase has a relatively high affinity for NH_4^+ in the presence of a medium containing potassium ions, whereas the affinity decreased almost 100-fold in sodium ion medium (*402*).

While it is possible that additional or alternative pathways exist for the biosynthesis of arginine and urea, the evidence advanced in support of these is on the whole not compelling. Even though the biosynthetic pathway to urea as outlined in its enzymatic steps still has many gaps to be filled before its function in an intact system can be completely evaluated, it rests on much sounder and more direct experimental evidence than the alternative pathways proposed.

Whatever modifications of the pathway may ultimately occur, it seems certain that the enzymatic steps outlined will continue to represent the major, if not the exclusive, pathway for arginine and urea biosynthesis.

E. Relation of Urea Biosynthesis to Pyrimidine Synthesis

Carbamyl aspartate has been shown to be a precursor of orotic acid, and thus of pyrimidines, in bacteria (*403–405*) and in animal tissues (*404, 406*). The mechanism of synthesis of carbamyl aspartate by carbamyl phosphate-aspartate transcarbamylase (also called aspartate carbamyl transferase, see *407*) is therefore of some interest. The enzyme occurs in animal tissues (*408–412*), microorganisms (*407, 412–415*) and plants (*416*), and catalyzes the reaction (80) shown.



$$\mathrm{H_2NC(=O)OPO_3^{2-} + HC(NH_3^+)(CH_2COO^-)COO^- \rightarrow HC(NHCONH_2)(CH_2COO^-)COO^- + HPO_4^{2-} + H^+} \qquad (80)$$

$\Delta F^{\circ\prime} < 0$

The enzyme, while specific for aspartic acid, also reacts with hydroxyaspartic acid (*417*). The distribution of aspartate transcarbamylase in rat (*410*), pigeon (*410*), and other animal tissues (*418–421*), and its distribution in liver of different species is shown in Table VI.

The wide distribution of the enzyme suggests that carbamyl aspartate is synthesized by most if not all tissues, and in all probability it represents the initial reaction in the synthesis of pyrimidines by these cells. This raises the question as to the nature of the carbamyl donor or donors in animal tissues other than liver. Although liver has been the only tissue in which the synthesis of carbamyl phosphate has been systematically investigated, there is evidence for synthesis of carbamyl phosphate in extracts of intestinal mucosa (*273*). Whether or not other tissues of mammals and tissues of nonureotelic animals can synthesize carbamyl phosphate remains to be demonstrated. All efforts in the authors' laboratory to demonstrate the presence of this enzyme in tissues other than liver and intestine have been consistently negative (*402*). Aside from the possibility that all animal tissues having aspartate transcarbamylase activity also have carbamyl phosphate synthetase activity, the following additional possibilities need to be considered: (*a*) carbamyl phosphate may be transported from liver to the extrahepatic tissues; (*b*) a carbamyl compound other than carbamyl phosphate may be synthesized in the extrahepatic tissues; and (*c*) a carbamyl compound other than carbamyl phosphate may be transported and converted to carbamyl phosphate. Citrulline could conceivably fulfill this role and has been reported to be capable of contributing its carbamyl group for carbamyl aspartate and pyrimidine synthesis (*422–424*).

While it is beyond the scope of this discussion to review the details of pyrimidine synthesis, it is of some interest to note that aspartate transcarbamylase activity is closely correlated with cell growth. Aspartate transcarbamylase tends to have the highest activity in those tissues which have a rapid cell turnover or growth. Thus the enzyme is high in ascites tumor cells of the mouse (*419*), primary and transplanted hepatomas (*418, 420*), growing plant root tips (corn, pea, sunflower, soybean, okra) (*416*), and regenerating liver (*420, 421*). In the latter system, which has been studied in some detail, the activity of aspartate transcarbamylase increases rapidly after partial hepatectomy and reaches a peak at about 36 hours, at which time it is about twice the activity of control tissue. The activity then decreases slowly and returns to normal levels after about 2 weeks. Coincident with the rise in aspartate transcarbamylase activity, there is a drop in ornithine transcarbamylase activity which slowly returns to normal levels after about 1 week (*425*). In addition, the activity of aspartate transcarbamylase of liver decreases from early embryonic stages of the rat progressively to that of adult

TABLE VI

DISTRIBUTION OF ASPARTATE TRANSCARBAMYLASE IN DIFFERENT ANIMAL TISSUES[a]

Tissue	Relative Activity[b]
Rat	
Intestinal Mucosa	200
Testes	110
Hepatoma	150–400
Liver	100
Regenerating liver (48 hrs. after hepatectomy)	200
Kidney	70
Lung	58
Spleen	42
Brain	40
Pancreas	38
Muscle (skeletal)	16
Muscle (heart)	2
Blood (whole)	2
Pigeon	
Intestinal Mucosa	124
Liver	106
Mouse	
Liver	44
Ehrlich ascites cells	400
Brain	12
Human	
Liver (Female, 18 yrs.)	24
Liver (Female, 48 yrs.)	12
Hepatoma (from above liver)	19

[a] Compiled from refs. *410*, and *418–421*.

[b] Specific activity, i.e. micromoles product per hour per mg. protein, of normal rat liver taken as 100.

levels (*426*). A relationship between aspartate transcarbamylase activity, pyrimidine biosynthesis and cell growth is thus suggested. In this connection Yates and Pardee (*414*, *415*) have presented evidence that in *E. coli* aspartate transcarbamylase synthesis is controlled by end product inhibition.

The relatively high aspartate transcarbamylase activity in regenerating liver and hepatomas is associated with a low activity of ornithine transcarbamylase and consequently of urea biosynthesis (*427*, *428*). We thus have here a case of biochemical dedifferentiation in which a highly specialized function, *viz*, urea synthesis, is lost in the case of the hepatoma and decreased in the case of the regenerating liver, but associated with an increase in the enzyme system, aspartate transcarbamylase, which is concerned with a more fundamental function, *viz.*, growth and proliferation. Carbamyl phosphate thus occupies a unique position in the liver in that, on the one hand, it can serve the highly differentiated and specialized liver cell for making arginine and urea and, on the other hand, it can serve as a precursor for pyrimidines. However, when the liver cell is dedifferentiated, as in the case of hepatoma, or in the early phases of regeneration, the need for pyrimidine synthesis is greater and the enzyme concerned with diverting carbamyl phosphate toward pyrimidine synthesis, *viz.*, aspartate transcarbamylase, is increased.

VIII. Summary Remarks

In surveying the enzymatic mechanisms available in animals, plants, and microorganisms, for the *production* of ammonia from organic compounds, one is impressed with the fact that all living cells share to a large extent the same enzymatic systems. Microorganisms and plants which have specialized systems for producing ammonia (e.g., nitrogen fixation, nitrate reduction, and aspartase) have these in addition to the more common ammonia-producing systems. A similar generalization would seem to be emerging in the case of systems *utilizing* ammonia. Glutamic acid dehydrogenase and glutamine (and asparagine) synthetase are apparently common to all classes of living organisms. Aspartase appears to be restricted to microorganisms and certain plants. The role of carbamyl phosphate synthetase or kinase still remains to be established as a third fundamental mechanism for ammonia utilization, although its role in ureotelic animals and certain microorganisms has been demonstrated.

For reasons already stated in this discussion, it is possible that carbamyl phosphate will emerge as a compound of fundamental importance in all living cells because of its role in the synthesis of arginine and pyrimidines. In addition, it has the unique feature of being formed as the result of a primary fixation of *both* ammonia and carbon dioxide and thus from the standpoint of biochemical evolution could represent one of the earliest biochemical compounds. Carbamyl phosphate perhaps deserves consideration, along with the many others proposed (*429*), as one likely to have been formed on the primitive earth, since it could conceivably have been formed readily by reaction of cyanate and phosphate. The synthesis is unique in the sense that it represents a direct synthesis of an organic compound from inorganic compounds and further represents a prototype of a high-energy phosphate bond compound.

The time of appearance of the enzymatic systems for the synthesis and utilization of carbamyl phosphate will have to await more extensive studies concerned with the formation and utilization of this compound in different living forms.

The time of appearance and rate of formation of carbamyl phosphate (carbamyl phosphate synthetase activity) has been studied in livers of rat and pig embryos (*430*) and liver of the tadpole (*324, 373*). Carbamyl phosphate synthetase is relatively late in its appearance in the embryo, being first demonstrable in the case of rat liver in the 16 day old fetus (0.8 gm. weight) and in the case of pig liver in the 28 day old fetus (2 cm. in length). The activity increases slowly up to parturition with a sharp increase occurring post partum, reaching the adult levels within 48–72 hours. The rate of increase of carbamyl phosphate synthetase activity in tadpole larvae is shown in Fig. 7. If ontogeny can be considered to be a recapitulation of phylogeny, it would appear that carbamyl phosphate synthetase was relatively late in its evolutionary appearance.

The widespread occurrence in microorganisms, plants, and nonureotelic animals of part or all of the enzymes and other components of the urea cycle, suggests that the mechanism which ultimately evolved represents an integration of component enzyme systems originally serving more restricted catalytic functions as separate systems. We thus have in the case of urea biosynthesis in true ureotelic animals an instance of a highly specialized function being acquired in the course of maturation of the embryonic liver cell. The factors other than genetic operating during embryogenesis which determine the time and sequence of appearance of the different enzymes making up the urea cycle are as yet unknown, but they are capable of experimental study. Studies by the authors with the metamorphosing tadpole are promising in this regard. The question of enzyme inducers, activators, etc., in the form of unique metabolites or special hormones, can be put to test experimentally with our present knowledge of the enzyme components involved in urea biosynthesis.

The continued survey of the occurrence of the enzymatic components of the urea cycle in plants, microorganisms and animals, along with the studies on embryogenesis, may be expected to reveal the evolutionary time-course and mechanism of development of the highly integrated pathways for the biosynthesis of pyrimidines, arginine, and urea.

Addendum

The data shown in Table VII appeared or were compiled after this manuscript was submitted.

TABLE VII

THERMODYNAMIC DATA FOR THE HYDROLYSIS OF ASPARAGINE AND GLUTAMINE[a,b]

Reaction	$\Delta F°$ (kcal.)	$\Delta H°$ (kcal.)	$T\Delta S°$ (kcal.)
Glutamine$^{\pm}$ + H_2O → Glutamate$^{\overset{-}{\mp}}$ + NH_4^+	−3.42	−5.16	−1.73
Asparagine$^{\pm}$ + H_2O → Aspartate$^{\overset{-}{\mp}}$ + NH_4^+	−3.6*	−5.71	−2.1*
Glutamine$^{\pm}$ + H^+ + H_2O → Glutamic acid + NH_4^+	−9.32	−6.11	3.2*
Asparagine$^{\pm}$ + H^+ + H_2O → Aspartic acid + NH_4^+	−8.9*	−7.24	1.7*

[a] Benzinger *et al.* (*431*).

[b] Temperature, 25°; values with asterisk have probable errors of 0.2 to 0.4 kcal.

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CHAPTER 5

Muscular Contraction

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I. Introduction

Many cells are capable of converting energy derived from metabolic processes into mechanical work without any apparent characteristic structure within which this particular function can be said to reside. This undifferentiated property of protoplasm is exemplified by the movement of ameba and of the leukocytes of vertebrates. When specialization occurs, however, fibrous structures are developed to carry out the contractile function, as for example in the protozoa, *Vorticella* and *Stentor*. In these organisms the contractile fibrils, the myonemes, show many of the properties associated with metazoan muscle cells. For example, contraction can be induced by electrical stimulation and in the case of *Vorticella* repeated stimulation produces a prolonged contraction or tetanus. Most authors have regarded the stalk of *Vorticella* as a simplified muscle; so much so that German workers referred to it as the "Stielmuskel" (stalk muscle) and often it was used for investigations of the physiological type. Recently Randall (*1*) has described the fine structure of the myonemes of certain ciliate protozoa.

The pattern of cells specialized for contraction evolved very early, and the two types which have emerged, the so-called striated and smooth muscle cells (Fig. 1), are widely distributed throughout metazoan life. Both kinds of cell contain large numbers of fibrous elements, the myofibrils, which are oriented in a parallel manner along the axis. The myofibrils of striated muscle have been intensively studied by a variety of techniques and there is no doubt that the events which convert the chemical energy provided by the cell into mechanical movement take place in these structures. Although smooth muscle is probably the simpler contractile system, much less investigation into its fine structure and biochemistry has been made, but from the evidence available and from analogy with striated muscle it seems most probable that filamentous structures which may or may not be organized into myofibrils are the contractile units of smooth muscle.

Striated muscle is distinguished from smooth by regular alternating light and dark bands which run across the cell at right angles to the axis. In addition to myofibrils both types contain the usual formed elements found in other cells, namely, nuclei, mitochondria [the granular components of muscle cells are often referred to collectively as sarcosomes (*2a*)], and membranous structures comparable to the endoplasmic reticulum of other cells, sometimes called the sarcoplasmic reticulum (*3, 4*). (See Fig. 2.)

Particularly among vertebrates striated muscles form a fairly homogeneous group in that these tissues consist almost entirely of muscle cells of the striated type and that histological, physiological, and pharmacological differences are small, not only between the different muscles of a given animal but also between representatives of all classes of vertebrates (*5*). Because of its homogeneity and general availability striated muscle has been widely studied and there is probably available more basic knowledge about the biochemistry of this tissue than of any other tissue of the body.

On the other hand, vertebrate tissues comparable to an anatomical muscle and consisting mainly of smooth cells, e.g., pilomotors in mammals and the retractor penis (*6*) in some species, occur only occasionally. Usually in vertebrates smooth muscle cells participate with various other tissues to form organs such as stomach, artery, uterus, iris, spleen, etc., in which contractility represents only one of the properties essential for the normal functioning of these organs. In further distinction from striated muscle, isolated vertebrate smooth muscle elements vary considerably in excitability, mediation, inhibition, and conductivity (*5*).

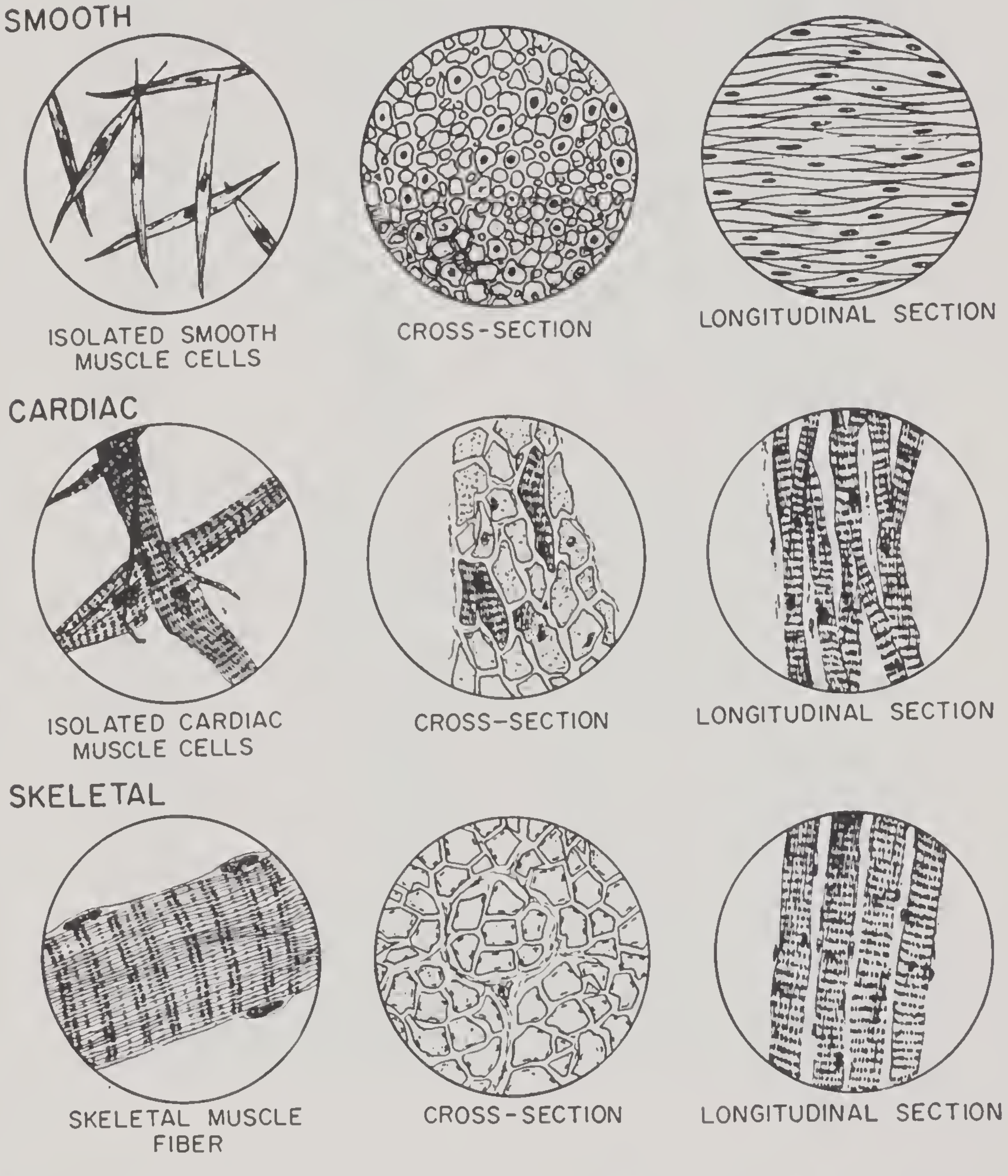

FIG. 1. Diagram to illustrate the relative size and the arrangement of cells in smooth muscle and the skeletal and cardiac forms of striated muscle. [After Kendall (2).]

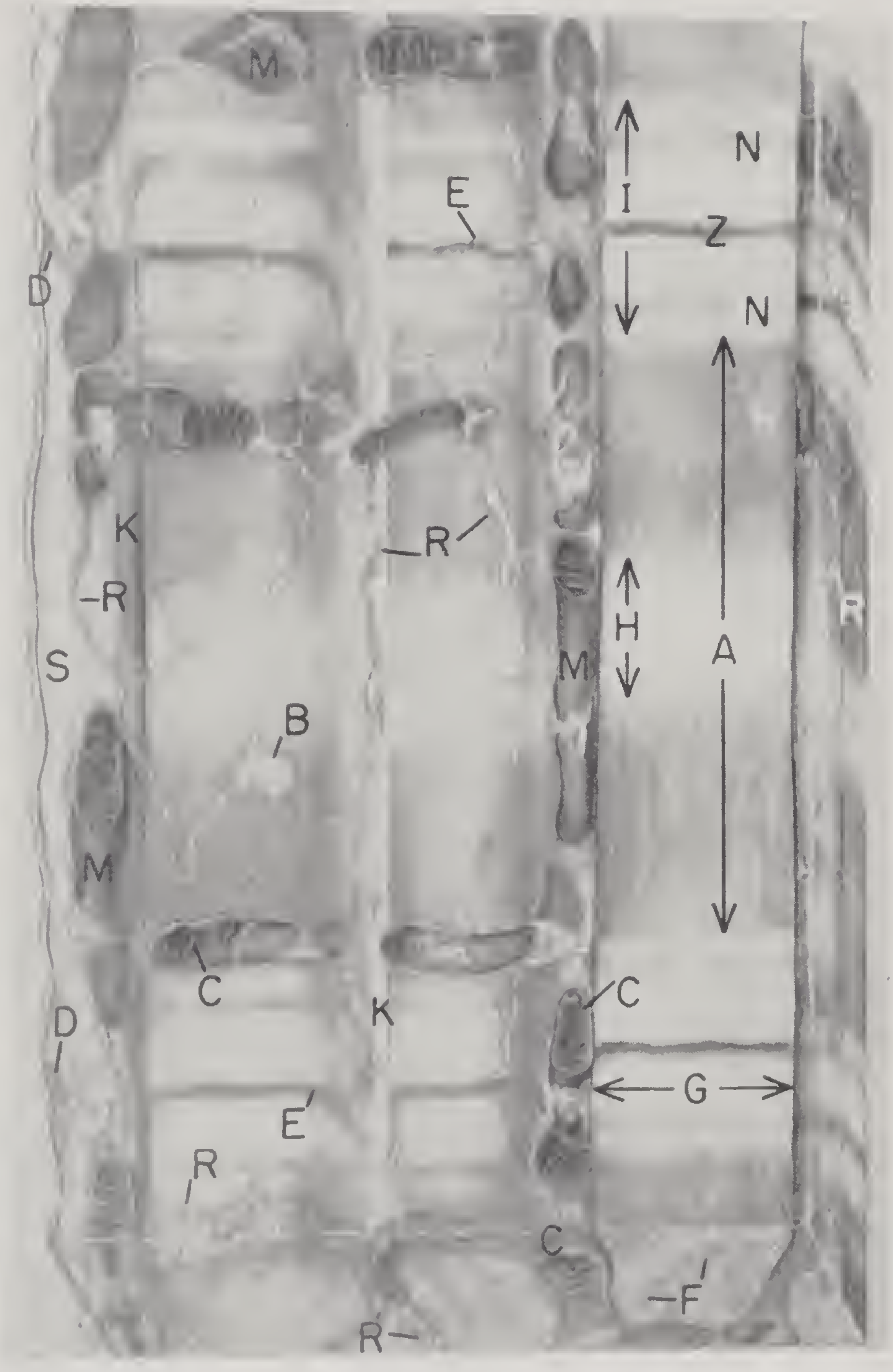

FIG. 2. Model of a block of striated muscle about 1 × 3 × 5μ. [After Bennett (*4*).]

KEY:

A, A band	H, H band
B, Cisternae	I, I band
C, Cristae mitochondriales	K, Sarcoplasm
D, Attachment of reticulum to sarcolemma	M, Mitochondria (Sarcosomes)
E, Attachment of reticulum to Z band	N, N band
F, Cut ends of myofilaments	R, Sarcoplasmic reticulum
G, Myofibril	S, Sarcolemma
	Z, Z bands

Vertebrate skeletal muscle is generally striated, whereas the visceral muscle is usually of the smooth variety and nearly always spontaneously active. Cardiac muscle of vertebrates constitutes an intermediate type in that although striated it is built up into a syncytium and shows spontaneous activity. Cobb (7) has pointed out the difficulty in certain cases of deciding whether a muscle is typically skeletal or visceral, a view which was endorsed by Ritchie (8) who took the stand that there are no fundamental differences between muscles but simply differences of degree. So far as the basic chemistry of muscle is concerned there is some support for this view. In the case of vertebrate tissues, however, according to Fischer (5) difficulties only arise in classifying muscle cells as smooth or striated during embryological development or in tissue culture. He takes the view that there is no histological or physiological evidence for the existence of an intermediate type of muscle cell. Even when striated muscle fibers are found distributed in smooth muscle coats, e.g., in the palatal membrane of fishes and in the whole gut of certain species (i.e., tench), the individual fibers behave physiologically and pharmacologically according to type.

In general striated muscle is less widespread in the invertebrates. The arthropods possess striated skeletal muscle, indeed insect skeletal muscle often shows very distinct striations, and unlike the vertebrates even their intestines are moved by striated muscle cells. Striated cells are well represented in the skeletal muscles of the crustacea, but among the mollusks muscles are composed mainly of smooth fibers, for example, the body muscles of the cephalopods and the adductors of the shell of the lamellibranchs, which are skeletal on morphological grounds. Likewise, the body muscle of the earthworm is also composed of smooth muscle cells. Striated muscle is found occasionally in the more primitive organisms, e.g., coelenterates have some striated fibers, but smooth muscle is characteristic of the lower phyla and is probably basically the more primitive type. Striations are indicative of more highly specialized tissue and it has long been recognized that striated muscles are usually of the faster kind, whereas smooth muscle has a "holding" function and is characterized by a slow rate of relaxation, a property which is particularly exemplified by some molluscan adductors (9).

II. Structure of the Myofibril

A. Striated Muscle

1. *Myofibrils of Vertebrates and Invertebrates*

In the living cell the myofibrils are not usually sharply defined and the cross striations appear to run continuously, but when the cell is mechanically disrupted the bulk of the cytoplasm breaks up into discrete fibrils. Thus *in situ* the myofibrils are held together so that their cross bands are strictly aligned with one another. The alternating relatively broad dark and light bands, which are apparent when the myofibril at relaxed length is viewed in ordinary light, are known as the anisotropic (A) and isotropic (I) bands respectively (Fig. 3). This difference in appearance between the bands is due to the fact that the A band is composed of material of higher refractive index. Further evidence of a basic difference in the molecular architecture of the A and I bands is that whereas the former exhibits a strong positive birefringence the I band is only weakly positively birefringent and is considered, for the purposes of nomenclature at least, to be isotropic.

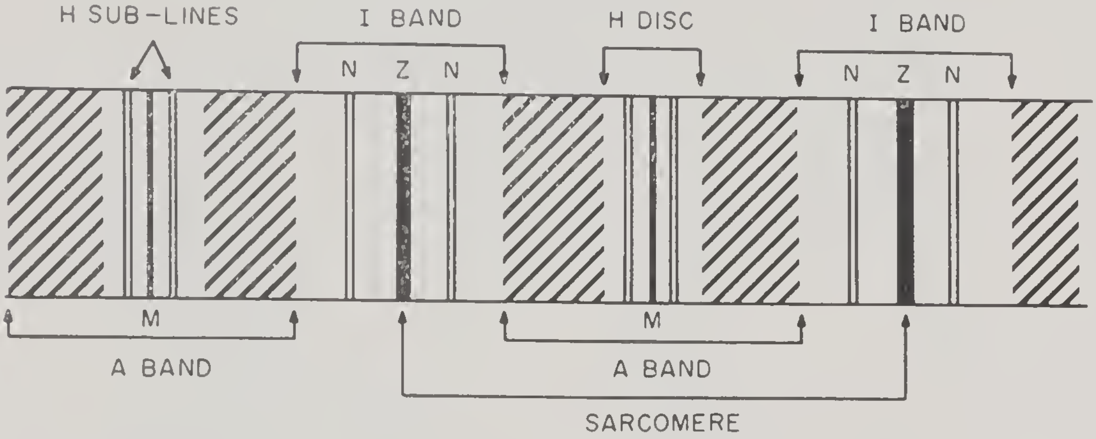

FIG. 3. Diagrammatic representation of cross bands and lines of the striated myofibril. It is not yet known for certain whether all the lines shown, e.g., H sublines, are present in the relaxed myofibril.

The general structural pattern of the striated (skeletal) myofibril appears to be common to all species in which it has been investigated. Differences have arisen in the description of the band changes which occur during contraction but these appear to be due either to differences in interpretation or to errors arising from the limitations of the techniques employed, rather than from basic differences in the striated muscles studied. Even today the band changes which occur during contraction have not been completely described but some measure of agreement appears to be emerging as to the general pattern (see Section IX).

The description of the myofibril given by the classical histologists has been confirmed and enormously extended by the application of the electron microscope to this study. Electron microscope investigations have been reported on the fine structure of striated muscle from the frog, *Rana temporaria* (*10, 11*); the toad, *Bufo marinus* (*12*); the rabbit (*13–17*); the domestic hen, *Gallus* sp. (*18*); the mouse (*19*); the golden hamster (*19*); the snake, *Constrictor constrictor amarali* (*20*);

the lizard, *Tupinambis teguixin* (*20*); the cayman, *Caiman latirostris* (*20*); the axolotl (*21*). Studies on invertebrate striated muscle by electron optical methods have been largely restricted to the phylum Arthropoda. The species investigated include the crab, *Paragrapsus quadridentatus* (*22*); the grasshopper, *Locusta migratoria* L. (*22*); the blackwater beetle, *Hydrophilus piceus* (*23–25*); *Hydrophilus ater* (*24, 26*); the water bug, *Belostoma* sp. (*26, 27*); the cockroach, *Periplaneta americana* (*26, 27*); the water beetle, *Dysticus marginalis* (*24, 26*); the wasp, *Pelopeus cementarius* (*28*); and the fire-fly, *Photurus pennsylvanica* (*28*); Diptera such as the blowfly, *Lucilia cuprina* (*29*) and the housefly, *Musca* (*28–30*).

The investigations cited by no means exhaust the literature but give some indication of the range of species studied. From these studies it is indeed striking that all the bands indicated in the diagram of the myofibril shown in Fig. 3 have at some time or other been demonstrated in skeletal muscle from both vertebrates and invertebrates. A, I, Z, and H bands are regularly seen in all types of relaxed skeletal muscle and the M line is also usually seen although Edwards and collaborators (*27*) report that in myofibrils from both flight and femoral muscles of *Periplaneta americana* the M line was poorly formed. Edwards *et al.* (*31*) claim that the M line is absent from insect slow muscle whereas Jordan (*32*) reports its presence in the myofibrils of the leg and elytra of the Japanese beetle, *Popillia japonica* Newman.

Less regularly observed features are the N lines, which have been reported as a single or a double line system in the I band on either side of the Z line. N lines have been reported as present in skeletal muscle from the toad, *Bufo marinus* (*12*), the domestic hen (*18*), and in cardiac muscle (*33*). Edwards and collaborators (*23, 31*) found the N lines weak or absent in crab and in high and low frequency insect muscles. Although it cannot be stated with certainty, the N lines appear to be present in the myofibril at the resting length, whereas the other less regularly observed feature of the myofibril, the H sublines (*12*) (called M sublines by Edwards and collaborators, *20, 23–27, 31*) lying in the H space on either side of the M line, are apparent or perhaps accentuated in the early stages of contraction. H sublines have been reported in toad, *Bufo marinus* (*12*), in the domestic rabbit (*14*), in reptiles (*20*), and in *Belostoma* sp. flight muscles (*26*). It seems likely that the inability of some workers to identify the finer cross striae of the myofibril is due to the fragility of these structures and the ease with which they can be obscured by other structures or lost during preparation. It is likely too that their distinctness may depend upon the particular stage of the contraction-relaxation cycle represented by the myofibril under examination. Bearing these facts in mind, the evidence available suggests that the cross bands and lines illustrated in Fig. 3 are all features of the structure of striated skeletal muscle wherever it is found and that they are reflections of the intricacy of the molecular pattern developed to carry out the rapid contraction-relaxation process which is characteristic of this kind of muscle (Plates I, II, and III).

Usually the myofibrils are oriented within the cell so that the cross striations run at right angles to the longitudinal axis of the cell. There is evidence that spiral striations exist in certain insects (*34*), molluscans (*35*), annelids (*36*), and ascidians. In the three latter groups the myofibrils are peripheral and the relative volume of sarcoplasm to myofibrils is high.

Although it is probable that the reported bands and lines are common to all striated skeletal muscles, certainly variations in size and intensity are well established and can be related to the type of function the muscle has to carry out.

In insect muscle, the striations are well marked and there is a wide variation in sarcomere length. Szekessy (*37*) showed that there is a good inverse correlation between sarcomere length and the frequency of contraction. His results, which are reproduced in Table I, indicate that the very fast wing muscles of flying insects have the shortest sarcomeres recorded (2μ or less) whereas the slower leg muscles average 4μ, and for the very slow mandibular muscle the period may be much greater. Edwards and collaborators (*31*) have confirmed these general findings from their electron optical studies and report sarcomere lengths of 10μ in termite mandibular adductor and 15μ in crab claw adductor muscles. Several exceptions to this rule have been pointed out (*25*), for example, the flight muscle of *Hydrophilus*, a powerful swimmer, has a shorter period than the leg muscle. Further *Hydrophilus* shows the same flight muscle structure as the Meliponidae, yet the latter are powerful flyers. Edwards and collaborators (*25*) conclude that the differentiation of muscle structure is more closely related to the phylogenetic position of the insect than to the specific muscle function. These workers (*31*) have also pointed out that the sarcomere length increases with the amount of shortening the muscle performs. In many of the electron optical studies of fast insect muscle the I band appears very much shorter than the A band—so much so that in some cases it is difficult to recognize distinct A and I bands (Plate II). Such an appearance is typical of many insect flight muscles in the electron microscope and it may be significant that during activity these muscles only shorten 2–4% (*31, 37a*). Certainly, as shown in the work of Brenner (*38*), the ratio of A band length to I band length is higher in the fast muscles of insects than in slow muscles.

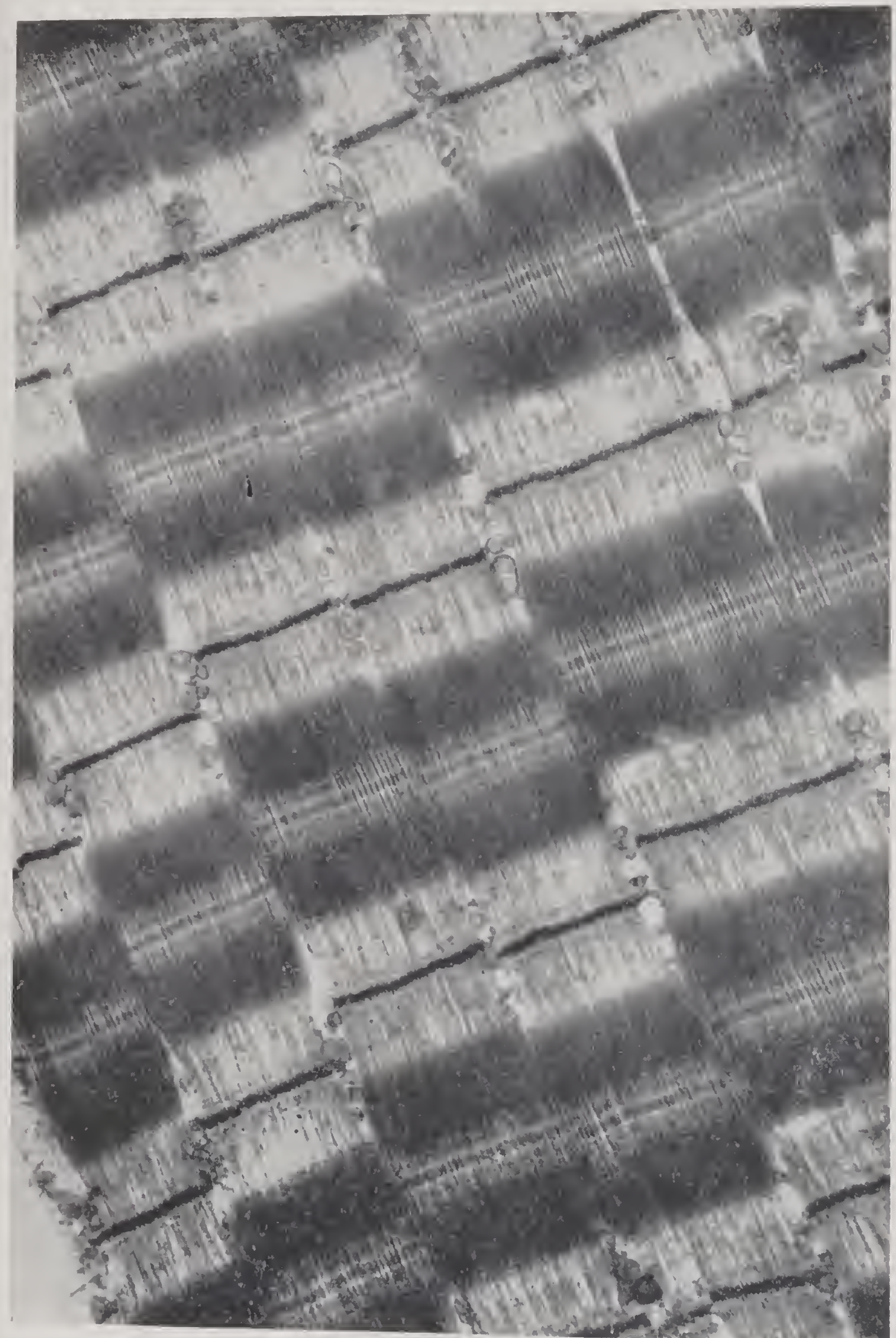

PLATE I. Electron micrograph of longitudinal section of rabbit psoas muscle showing small sarcosomes mainly in the region of the I band. A band = 1.5 μ. (By H. E. Huxley.)

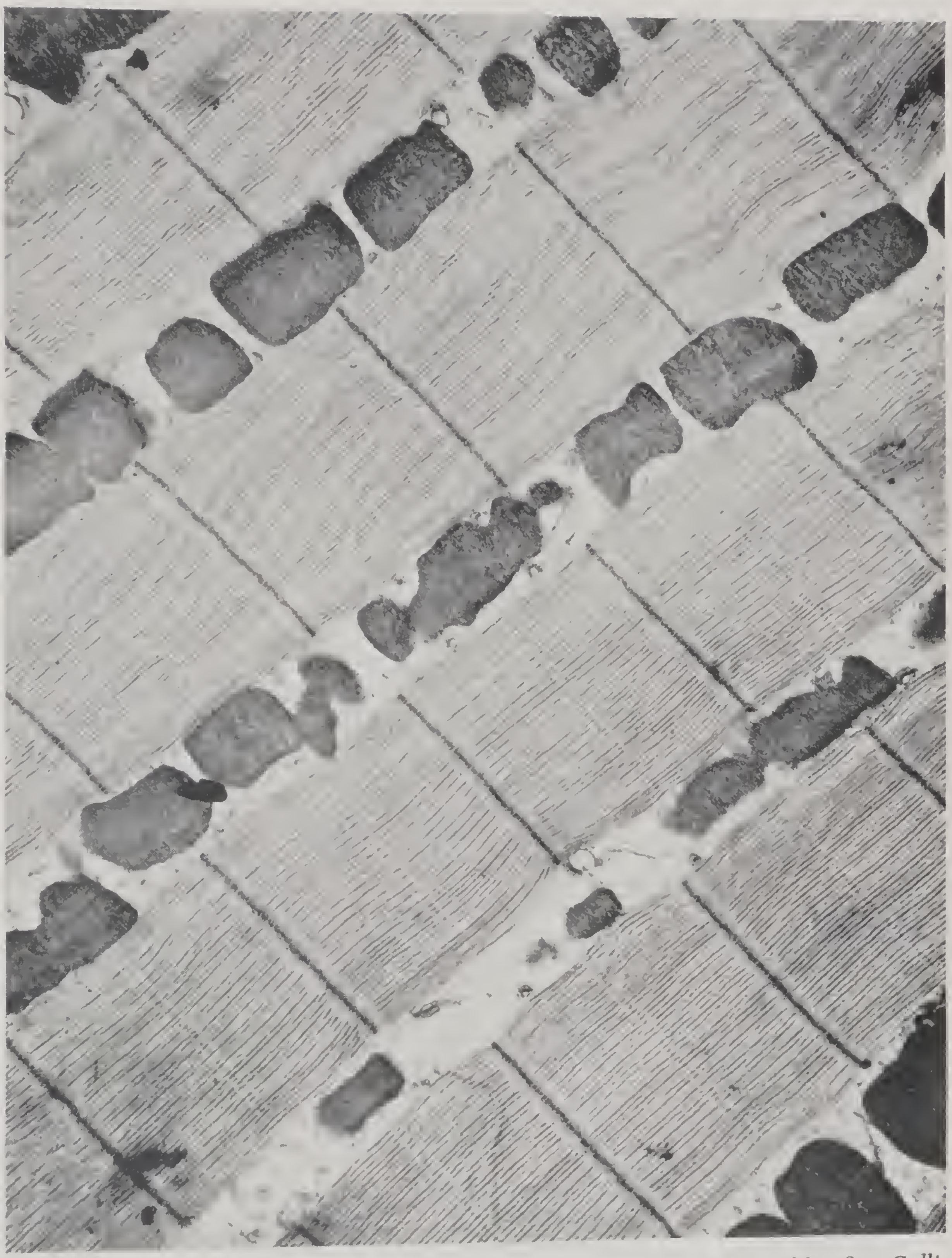

PLATE II. Electron micrograph of longitudinal cross section of blowfly, *Calliphora,* flight muscle. The myofibrils are characterized by very short I bands and the sarcosomes are large. A band ~3 μ. (By H. E. Huxley.)

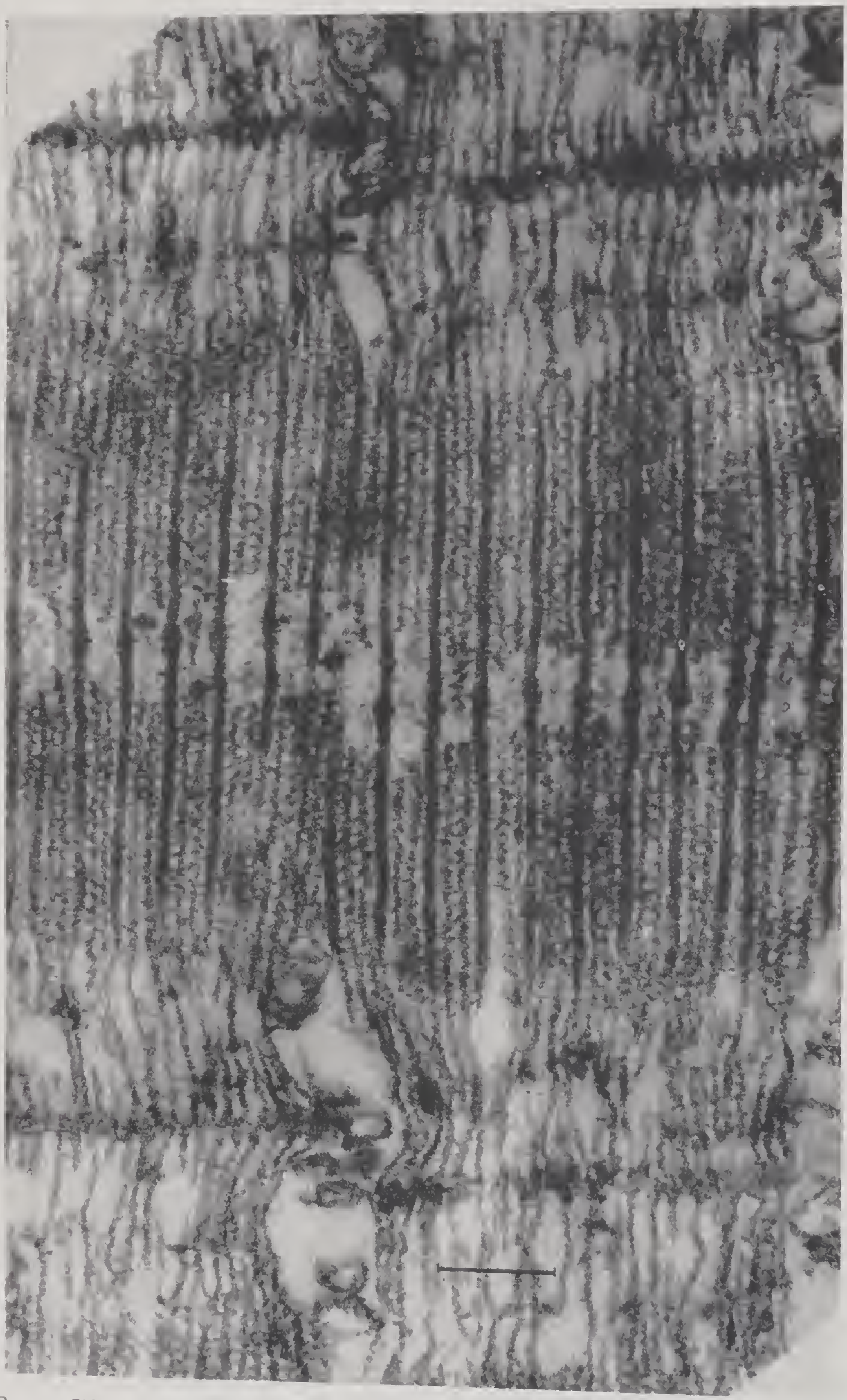

PLATE III. Longitudinal section through one sarcomere of myofibril from rabbit psoas muscle. The primary (A) filaments are somewhat thickened in the H band but elsewhere their diameter is ~100 Å. The diameter of the secondary (I) filaments is ~50 Å. Magnification mark, 0.1 μ. [From H. E. Huxley (*40*).]

Recent figures for the resting length of sarcomeres from vertebrate skeletal muscle are: rabbit, 2.3μ (A = 1.5μ, I = 0.8μ) (*17*) and frog, 2.5μ (A = 1.5μ, I = 1.0μ) (*39*).

TABLE I

SARCOMERE LENGTHS (IN MICRONS) OF STRIATED MUSCLES FROM INSECTS[a]

Name of Insect	Musculus adductor mandibulae	Musculus extensor tibiae	Musculus dorso-ventralis	Remarks
1. *Decticus verrucivorus* ♂	5.4	2.7	2.0–2.7	Wings unfit for flying; jumper legs
2. *Locusta viridissima* ♀	5.4	2.7	2.7	Larva, unable to fly, jumper legs
3. *Stenobothrus* sp.	—	1.4–2.7	—	Larva, jumper legs
4. *Liogryllus campestris*	5.4	4.0–5.4	2.7	Jumper legs
5. *Carabus scheidleri jucundus*	2.7–4.0	5.4	5.4	No wings
Carabus scheidleri jucundus	5.4–8.1	5.4	4.0	No wings
6. *Cybister laterimarginalis*	5.4–6.7	2.7–4.0	2.0–2.7	Swimmer legs
Cybister laterimarginalis	4.0–6.7	2.7–4.0	2.7	Swimmer legs
Cybister laterimarginalis	5.4–8.1	4.0	2.0–2.7	Swimmer legs
7. *Hydrous piceus*	8.1–10.8	5.4	4.0	
Hydrous piceus	5.4–8.1	5.4–6.7	1.3–2.7	
8. *Gnaptor spinimanus*	5.4	5.4	4.0–5.4	No wings
9. *Anoxia orientalis*	8.1	3.5–5.4	—	
10. *Lucanus cervus* ♂	6.8–8.1	4.0–5.4	1.7	Big
Lucanus cervus ♂	8.1	5.4	2.0–2.7	Medium size
Lucanus cervus ♂	8.1	5.4	1.3–1.7	Small (ab. capreolus)
Lucanus cervus ♂	8.1–10.8	5.4	1.0–1.5	
11. *Cerambyx cerdo* ♂	5.4	4.0	1.3–2.7	
Cerambyx cerdo ♂	5.4	5.4	1.3	
12. *Sphinx convolvuli*	/5.4/[b]	2.7–4.5	1.3–1.7	
13. *Apis mellifica*	—	2.7–4.0	2.0–2.7	
Apis mellifica	—	2.7–4.0	2.7–4.0	
14. *Polistes gallica*	5.4–6.7	2.7–4.0	2.7–4.0	
15. *Bombus lapidarius*	—	4.0–4.5	2.0	
16. *Aedes vexans*	—	1.7	1.7	
Aedes vexans	—	1.5–2.0	1.7	

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[a] After Szekessy (*37*).

[b] Musculus dilatator pharyngis used in this instance.

Most of the measurements of myofibril diameter fall in the range 0.5–2.0μ irrespective of the source of the skeletal muscle. It appears that variation can occur within a given cell as well as between myofibrils from different sources. Differences in the reported figures for the myofibril diameter can also occur as a result of inconsistencies in the shrinkage occurring during fixation and the difficulty often experienced in breaking myofibril bundles into single units. It is not surprising therefore that as yet no correlation has been made between diameter on the one hand and function or evolutionary development on the other, indeed if any correlation exists. As the myofibril is dependent on the sarcoplasm for the supply and removal of metabolites, it is more likely that its diameter is determined by purely physical considerations governing the effective penetration and the removal of these substances to and from all points in the interior. The myofibrils of red (rapid) insect muscle are often more clearly separated and more uniform in diameter than myofibrils from the white (slow) muscle (*24, 27*), which often form a synfibrillar continuous system (*24*).

2. *Fine Structure of the Myofibril*

Study of longitudinal sections of myofibrils from many species by the electron microscope reveals that these structures are built up of fine protein filaments, myofilaments, running parallel to the axis. The precise form and arrangement of the filaments is a matter of some controversy. The most detailed analysis has been carried out by H. E. Huxley (*15, 40*), who on the basis of high resolution cross section studies concludes that the myofibril of rabbit skeletal muscle is built up of

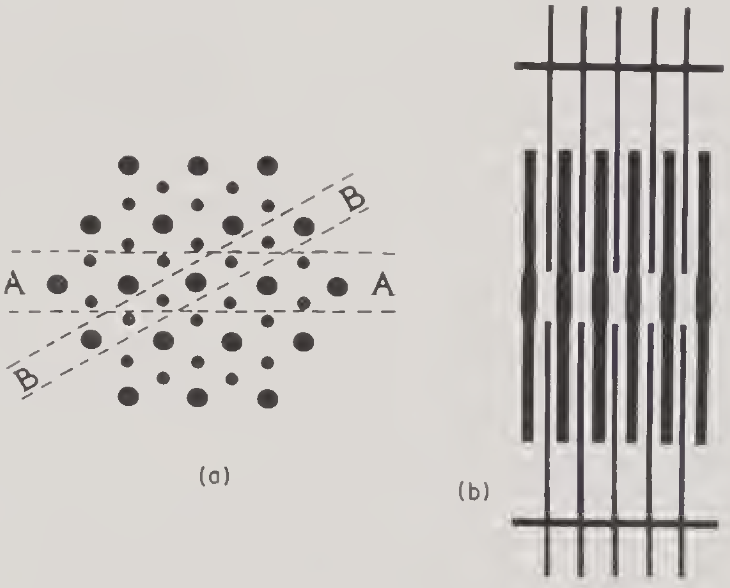

FIG. 4. Diagram of the structure of the myofibril proposed by Huxley (*40*): (*a*) Cross section showing double hexagonal array of filaments in the A band. A-A represents the direction of the longitudinal section illustrated in (*b*). An electron micrograph of a longitudinal section made in the direction B-B is illustrated in Plate III.

two types of filament. The A filaments, 110 Å in diameter, run continuously throughout the A band thickening to 140 Å in the area of the H space. The I band consists solely of the I filaments, 40 Å in diameter, which run continuously into the A band up to the edge of the H space. In the region of the A band where the two types of filaments overlap they form a twofold hexagonal array (Fig. 4).

The Huxley model for the structure of the myofibril is not as yet universally accepted for some investigators consider that the myofilaments run continuously through all bands of the sarcomere. Certainly in many published electron micrographs this appears to be so, although it is possible that this appearance is due to the fortuitous aligning of interdigitating sets of filaments. The only other detailed high resolution studies of striated myofibril cross sections so far published is that carried out by Hodge (29) with the blowfly (*Lucilia cuprina*) flight muscle. Hodge has interpreted his findings on the basis of the model illustrated in Fig. 5 which represents the myofibril as consisting of continuous myofilaments possessing a thick core which is less electron-dense than the thinner cortex. This cortex is considered to thicken up in the regions of the A and M bands to give the characteristic dense appearance in these regions. Earlier, Farrant and Mercer (22) reported the presence in myofibrils from crab leg and grasshopper wing muscles of similar filaments possessing a central core of 160 Å enveloped by denser sheath 40 Å thick. These workers came to the conclusion that such filaments ran continuously through the sarcomere and bands were formed by distribution of accessory protein along them. From the study of longitudinal sections of rabbit psoas and chick leg muscle, Spiro (41) is of

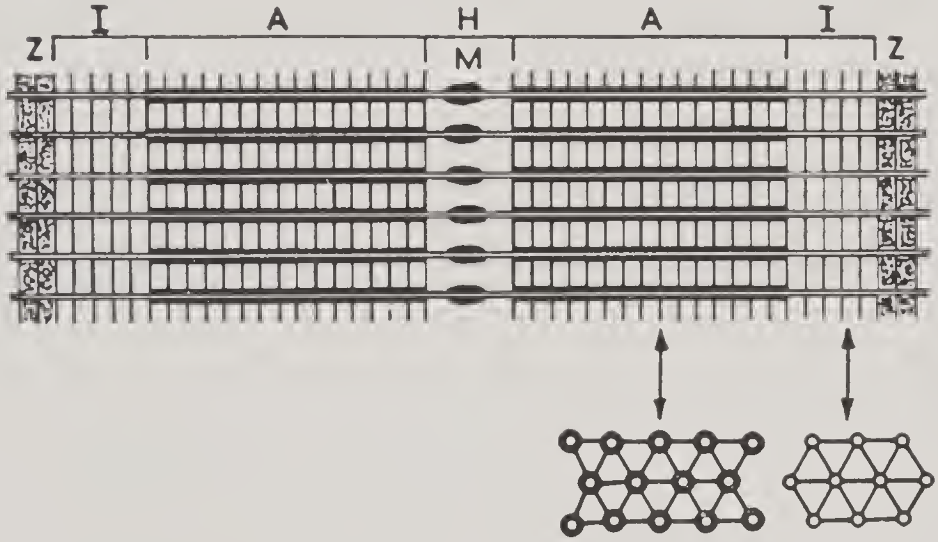

Fig. 5. Scheme proposed by Hodge (29) for the fine structure of the blowfly flight muscle myofibril. Transverse sections through the A and I band are shown at the bottom right.

the opinion that, in the A band other than the part occupied by the H space, filaments are about 60–70 Å in diameter and approximately twice as numerous as the thicker filaments found in the H space. He suggests that several thin filaments unite to form the thicker filaments in the H space. Like Hodge, Spiro concludes that all the A filaments run continuously through the I band to the Z band, the increased density of the A band being due to interfilamentous protein, presumed to be myosin.

Very recently, Huxley (*40*) has reinvestigated the problem of the nature of the filaments of rabbit skeletal myofibrils and has been able to demonstrate both thick and thin filaments in thin longitudinal sections of the A band. By taking such longitudinal sections at different angles he has obtained electron micrographs showing thick filaments in some cases separated by two distinct thin I filaments (see Plate III) as would be predicted on the basis of the twofold interlocking hexagonal array originally proposed earlier (see Fig. 4, *a*).

Many investigators have observed particularly in the A band a regular longitudinal periodicity along the myofilaments. The reported values range from 250 Å to 400 Å and there is some evidence that the periodicity is related to the degree of contraction (*12*), the number of such periods per sarcomere remaining constant. In high resolution studies (*40*) with thin sections such periodicity along individual filaments is not apparent but cross links between A and I filaments occur approximately every 400 Å. It is possible that such structures are in some way related to the periodicity observed in early studies.

The results of Huxley's investigation are rather convincing, particularly as he has attempted to explain why, under certain sectioning conditions, electron micrographs can be obtained which give misleading impressions of the myofibrillar structure (*40*). It is unlikely that fundamental differences exist in the ultrastructure of vertebrate and invertebrate muscle, but further investigation alone will decide if the reported differences are due to differences in technique and interpretation. Clearly an accurate description of the fine structure of the myofibril is an essential basis for any hypothesis which is proposed to explain the mechanism of contraction.

B. Smooth Muscle

1. *Vertebrate*

The fibrillar elements of smooth and embryonic muscle are much less well-defined than those of the striated type and in the past considerable attention has been paid to this aspect of smooth muscle cytology (Plate IV) [see Mark (*42*) for review of some of the literature]. Under appropriate conditions, smooth muscle can be teased out into myofilaments which vary greatly in diameter (*42a*). For example, in the urinary bladder of the frog all filaments are 50 Å in diameter, whereas from the same tissue in the mouse they are 100–200 Å thick (*42b*). In the gut of the turtle and chicken the myofilaments range in diameter from 150 to 230 Å, and exhibit a longitudinal repeating period of 400 Å, but no structures comparable to paramyosin fibrils (see Section IV,D,3) have been observed (*43*). Myofilaments 100–200 Å in diameter have also been reported in human and rat uterine smooth muscle cells (*42*).* In the latter study, longitudinal periodicity was not observed and it appears that the myofilaments are not clearly organized in discrete regular bundles as in the striated muscle cell but appear to fill the whole of the cell as a single loose bundle within which there are large and small irregular clefts containing the nucleus, mitochondria, and other cytoplasmic components (*42, 42a*). The myofilaments are not strictly aligned parallel to one another and, in consequence, cross sections do not exhibit the regular hexagonal pattern which is so characteristic of striated muscle.

2. *Invertebrate*

Considerable variation in function and morphology is found among the smooth muscles of invertebrates and in many cases, e.g., the earthworm, this tissue performs similar functions to those associated with striated muscle in the vertebrates. Although numerous light microscope studies are reported in the literature, details of the fine structure of invertebrate smooth muscle, like its vertebrate counterpart, are scanty (Plates V–IX). Fibrils (paramyosin fibrils) with characteristic fine structure very different from the striated myofibril have been isolated from foot and adductor muscles of mollusks. They are composed in part at least of protein apparently identical to the insoluble tropomyosin isolated by Bailey (*44, 45*) and Kominz *et al.* (*46, 47*) from these tissues. (See Section IV,D,3 on paramyosin.) There is evidence that the paramyosin structure occurs in muscles other than molluscan adductors, e.g., earthworm body-wall muscle (*42a, 47a*).

One type of smooth muscle fiber, found in platyhelminths, nematodes, annelids, sipunculids, echinoderms, and mollusks, exhibits a cross lattice of fine lines running obliquely to the axis, which is often referred to as "double oblique striation" [see Hanson (*36, 42a*) for references]. It has been suggested by Marceau (*35*) and others that this ap-

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* Shoenberg (*43a*) reports only one type of filament about 50 Å thick in rabbit uterus muscle.

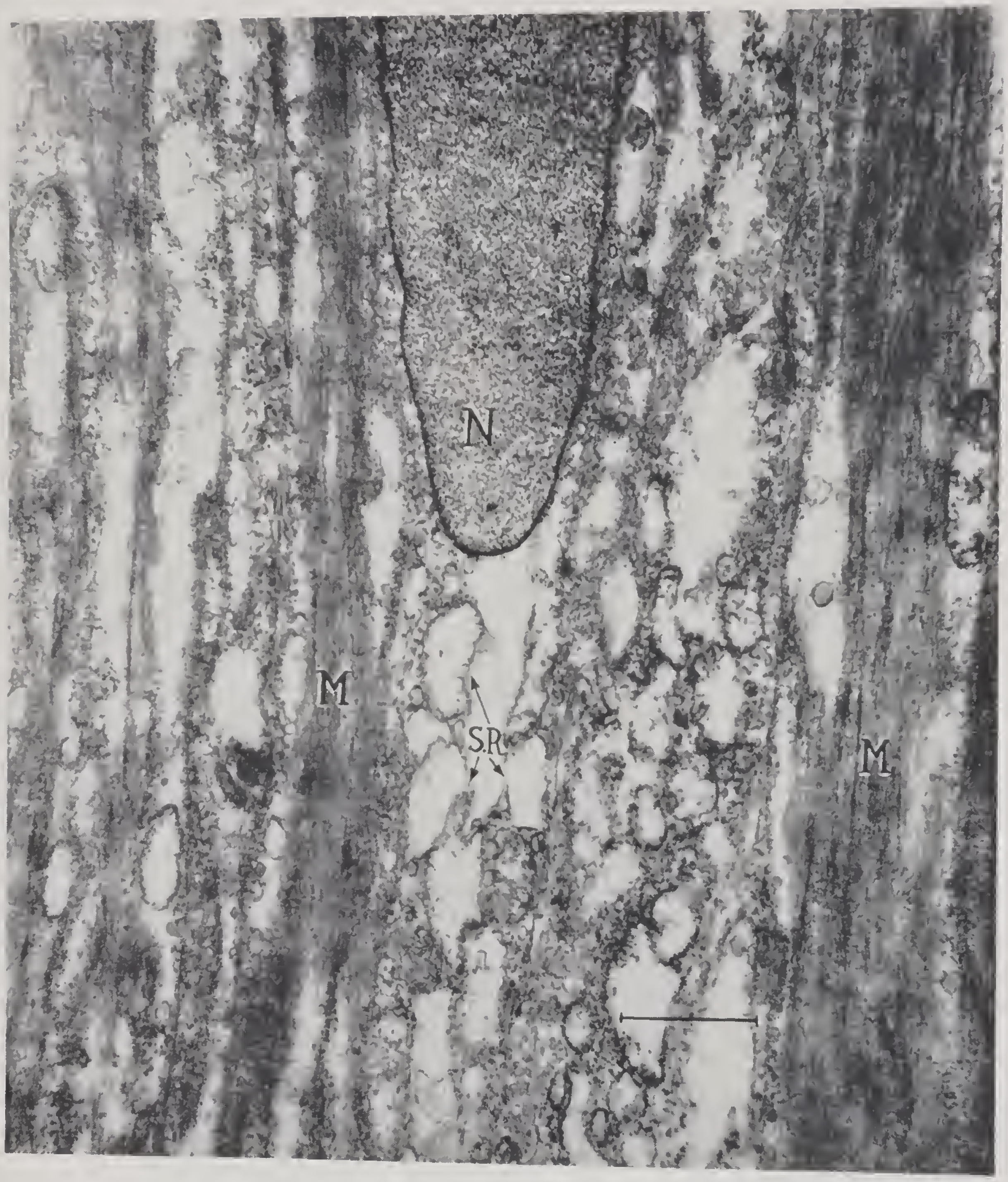

PLATE IV. Electron micrograph of portion of a smooth muscle cell from the pigeon crop. Sarcoplasmic reticulum (S.R) with numerous large vesicles, can be seen at the apex of the nucleus (N). Bundles of myofilaments (M) are interspersed in the sarcoplasmic reticulum. The myofilaments are of one type 50–70 Å in diameter. Fixed in buffered osmic acid after 3 days glycerination. Magnification mark, 1.0 μ. (By C. F. Shoenberg, unpublished.)

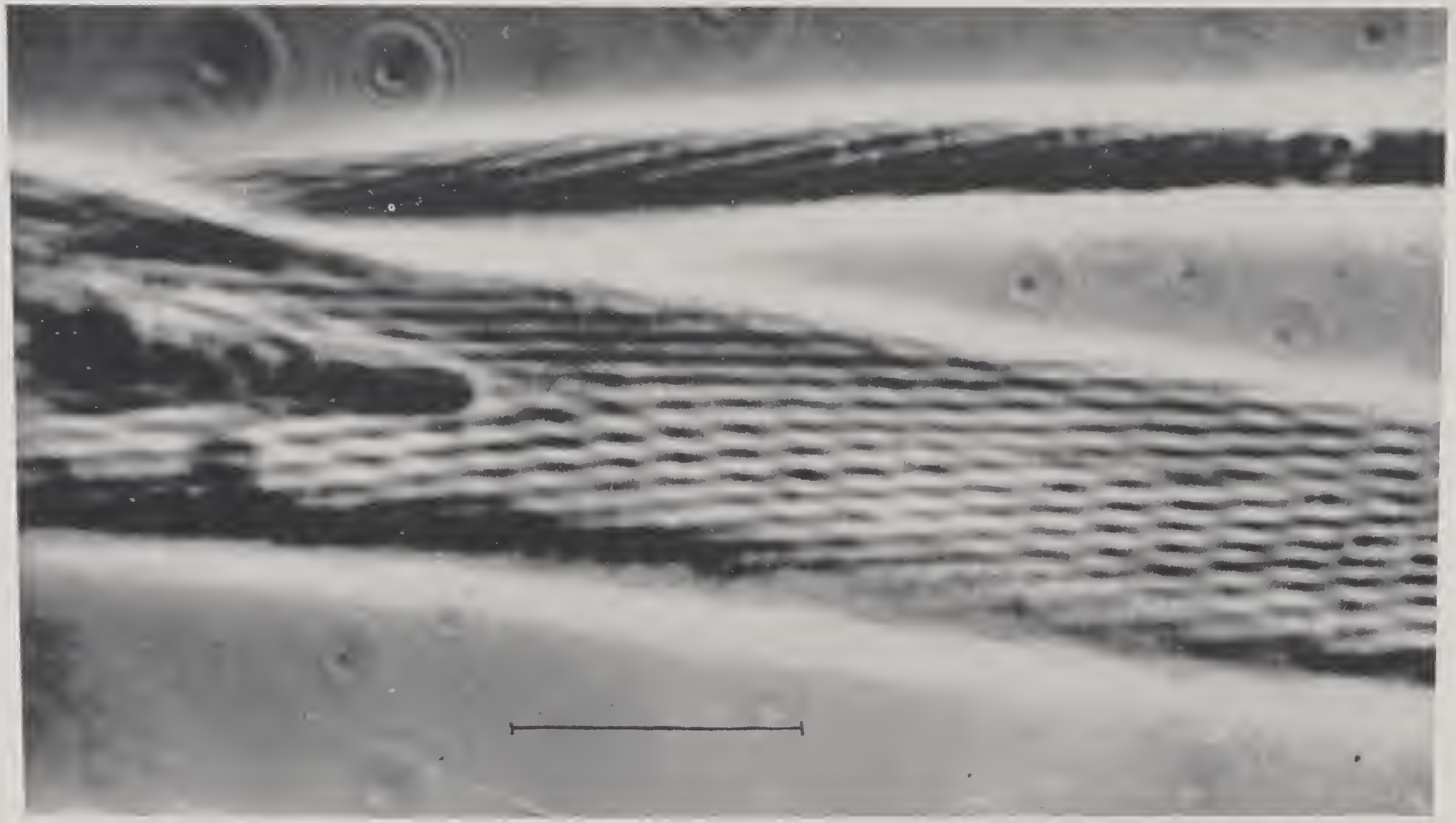

PLATE V. Light micrograph taken with phase contrast of a single smooth muscle cell isolated from the mantle of the squid. The lattice appearance is due to the helical arrangement of the myofibrils which are themselves unstriated. At one end of the picture the nucleus can be seen. Formaldehyde fixed. Magnification mark, 10 μ. (By Hanson and Lowy.)

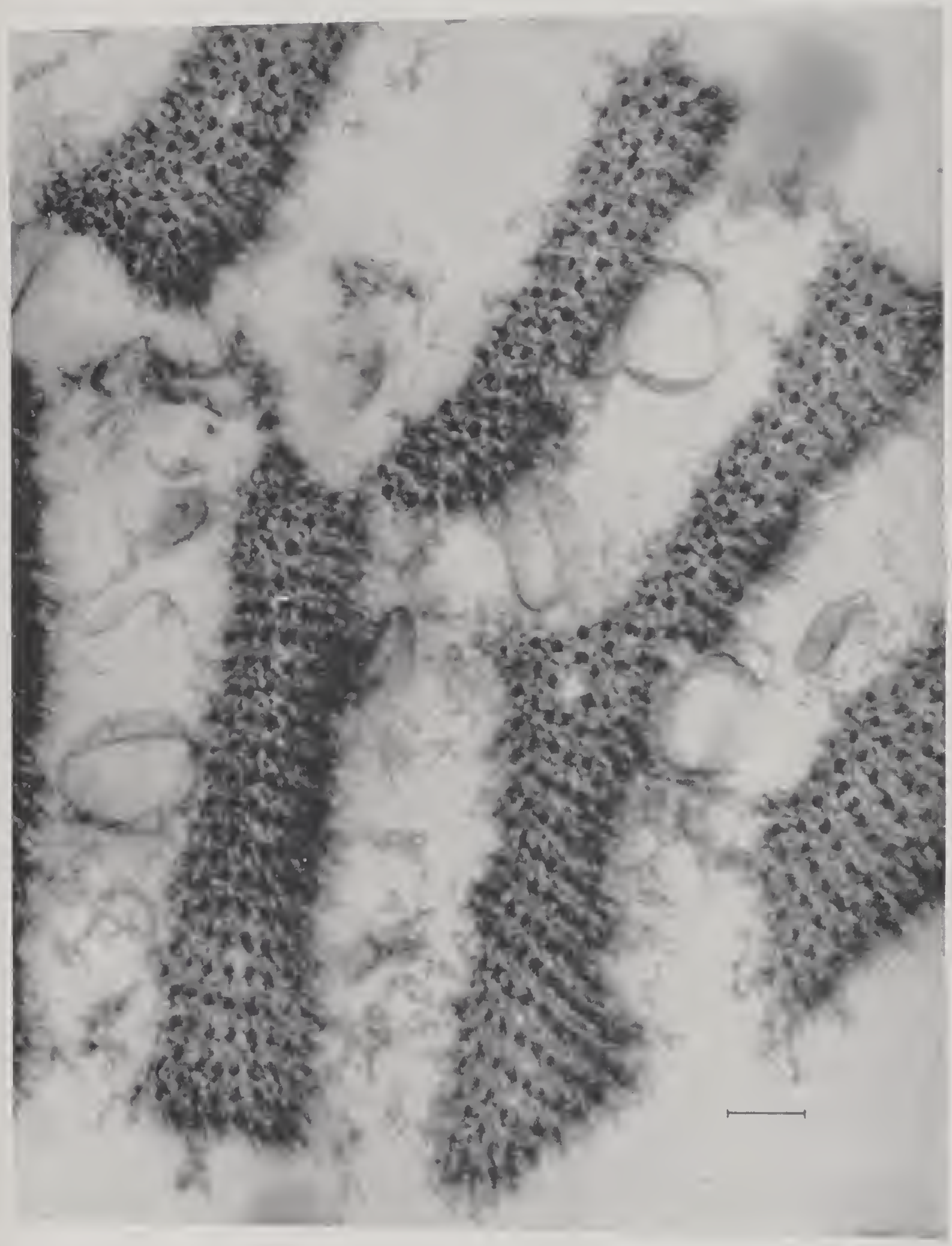

PLATE VI. Electron micrograph of a transverse section through a group of myofibrils in a smooth muscle cell of the squid funnel retractor muscle. The main myofilaments are 100–140 Å in diameter and are regularly arranged. Thin filaments of ~50 Å can be seen between the main filaments. Glycerol extracted and fixed with osmic and phosphotungstic acid. Magnification mark, 0.1 μ. (By Hanson and Lowy.)

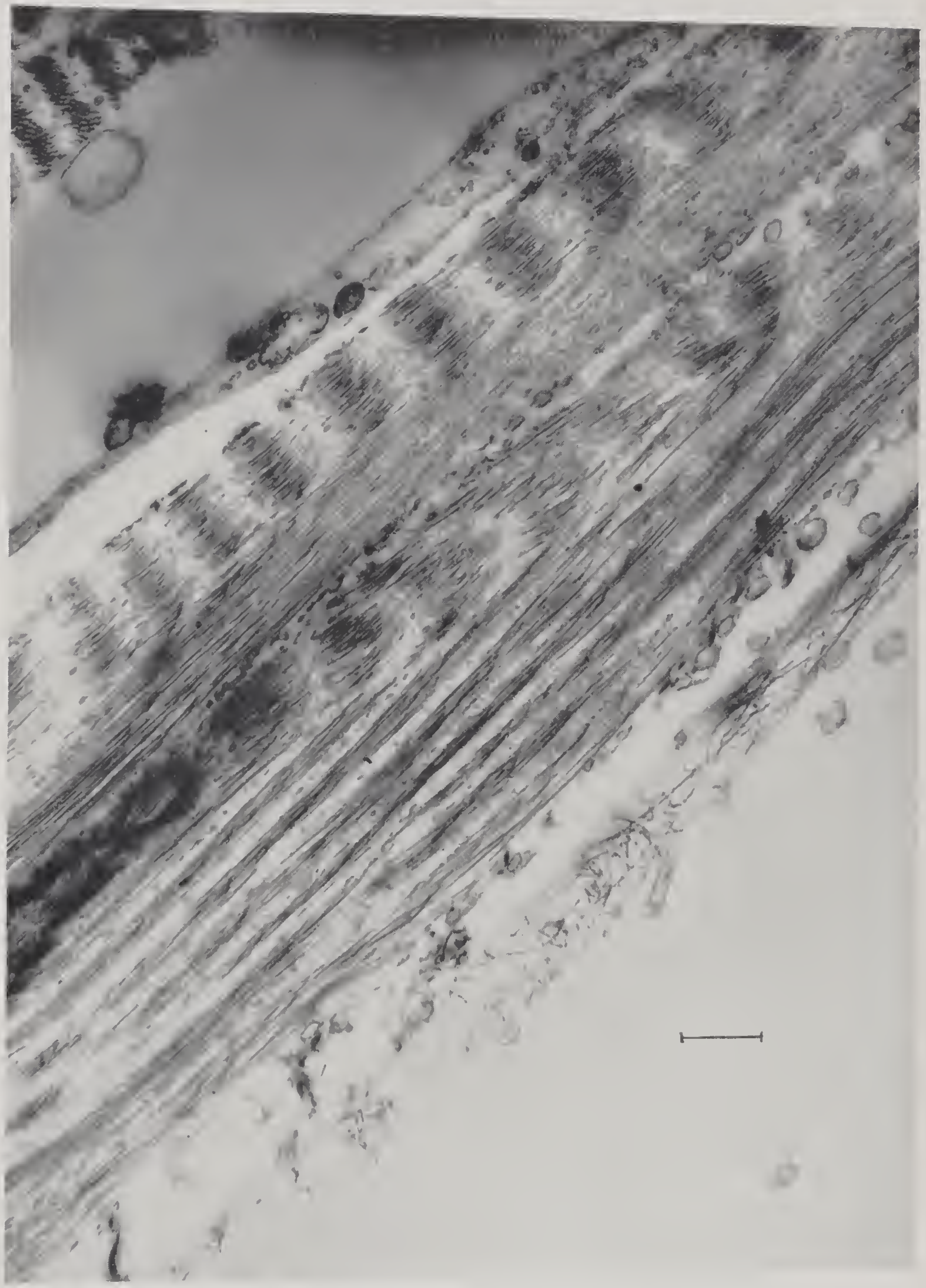

Plate VII. Electron micrograph of a longitudinal section through two cells (large vacuoles mark the cell boundaries) from the longitudinal smooth muscle coat of the earthworm. The section is parallel to the cell axis but in some places the myofibrils are cut obliquely due to their helical arrangement in the cell, and in consequence certain regions of the photograph show false striations. It can be clearly seen that striations are absent in regions where the myofibrils are parallel to the plane of sectioning. Elements of sarcoplasmic reticulum can be recognized between the myofibrils. Glycerol extracted and fixed with osmic and phosphotungstic acids. Magnification mark, 1 μ. (By Hanson.)

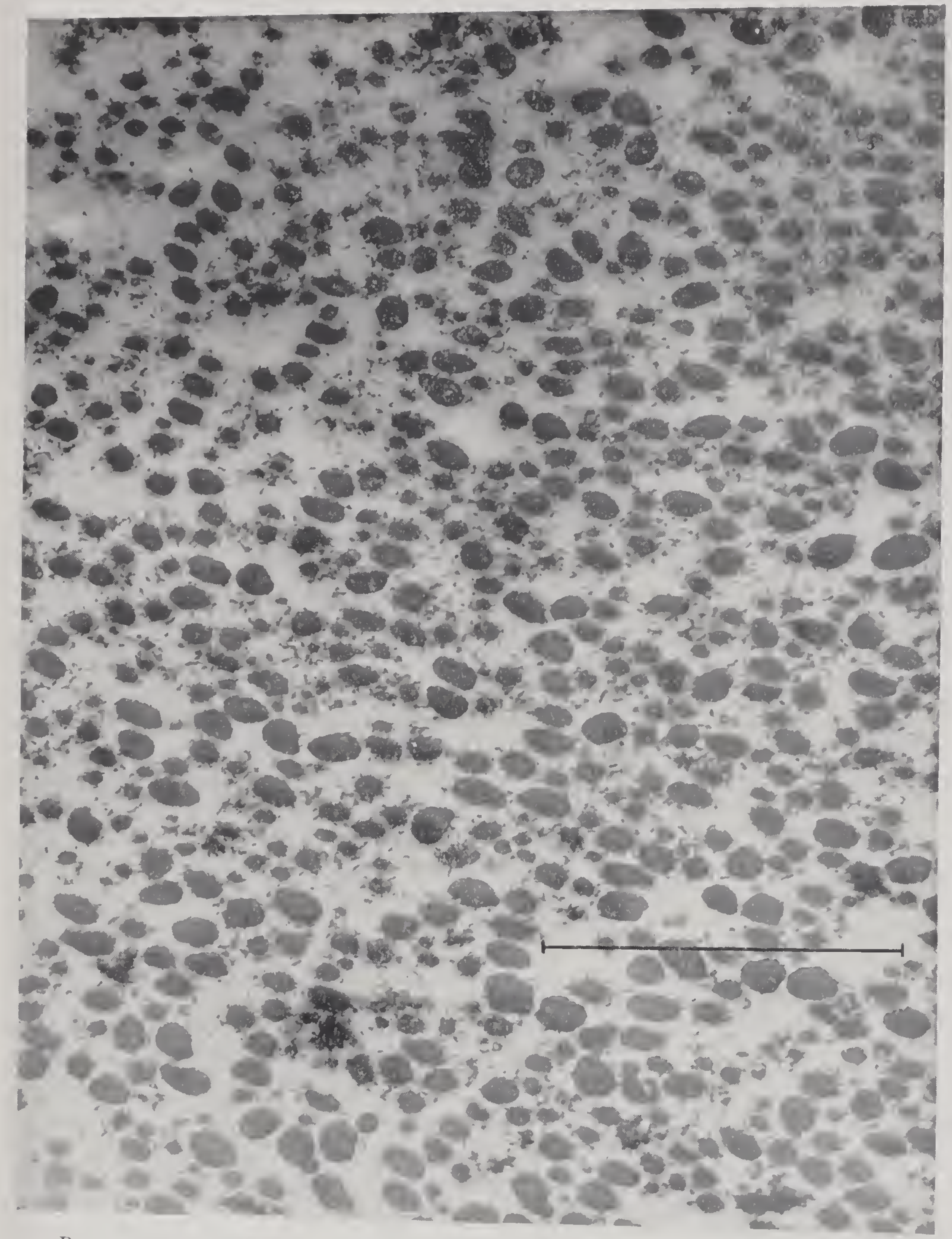

Plate VIII. Electron micrograph of transverse section through a fiber of the white (tonic) part of oyster adductor muscle. Filaments vary in size up to 1500 Å, are randomly arranged and myofibrils are not apparent. These filaments are made largely of paramyosin. Fixed in formaldehyde and phosphotungstic acid. Magnification mark, 1 μ. (By Hanson and Lowy.)

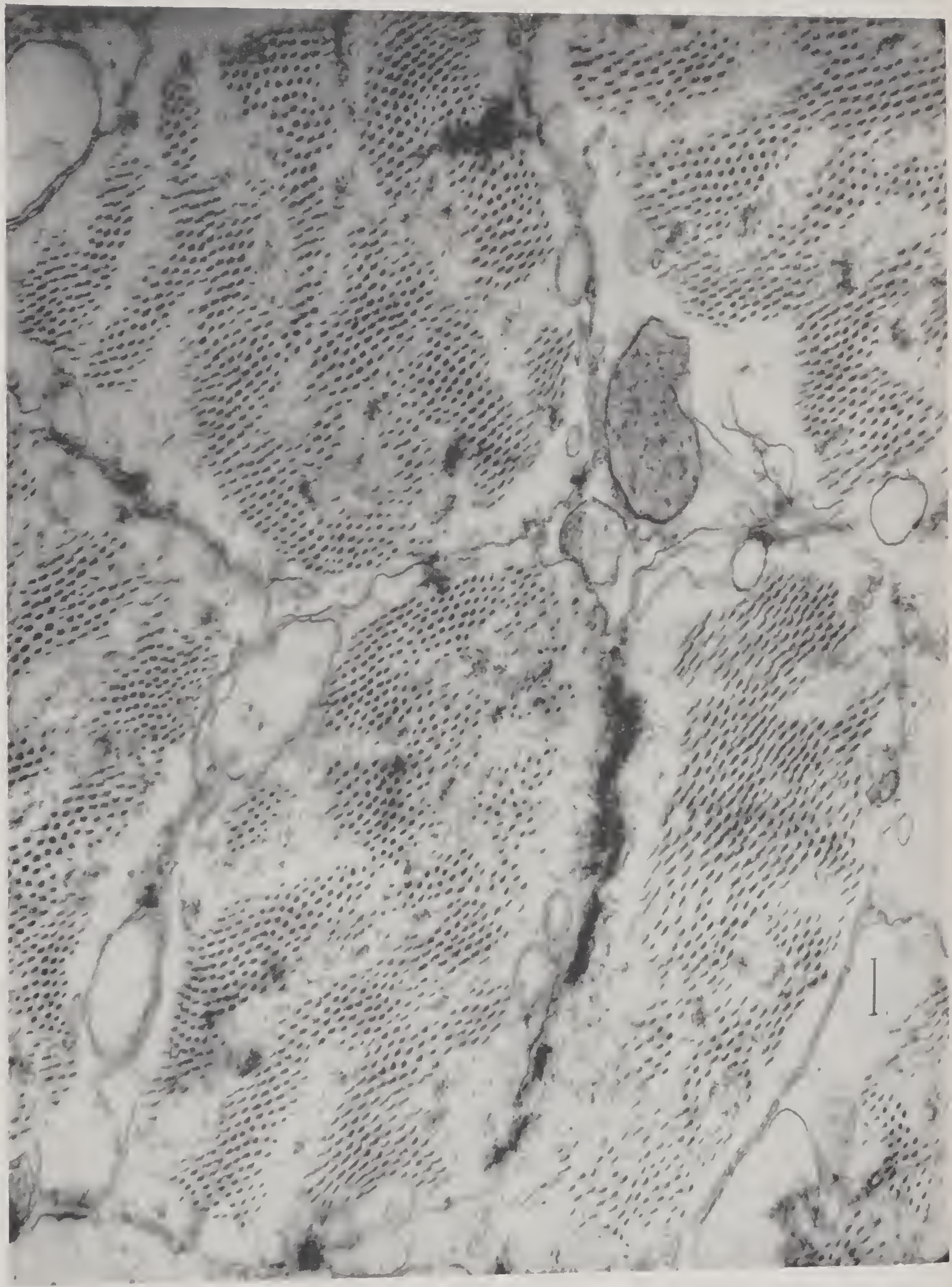

Plate IX. Electron micrograph of transverse section through the yellow (tinted, used for rapid contraction) part of oyster adductor muscle. The filaments are smaller than those in the white portion; they are mostly of the same size, regularly packed, and tend to form myofibrils. The fibrils are arranged helically and planted in a complicated manner within the cell. Fixation in osmic and phosphotungstic acids. Magnification mark, 1 μ. (By Hanson and Lowy.)

pearance arises from helically arranged fibrils within the smooth muscle fibers. Hanson (*36*) has recently examined the smooth body-wall muscle of the earthworm *Lumbricus terrestris* L. with the electron microscope and gives some details of the nature and arrangement of the fibrils within such fibers (Plate VII). Her findings are summarized in Fig. 6. Although the precise helical arrangement which gives rise to the "double oblique striation" was not analyzed in this investigation it was shown that the fibrils, like the cell itself, have a ribbon-like form, the greatest width being about 2μ with a thickness of 0.2μ. Each fibril lies at an angle to the fiber axis and on contraction this angle increases. Seen in cross section, the thin side of the myofibril is close to the cell surface while the

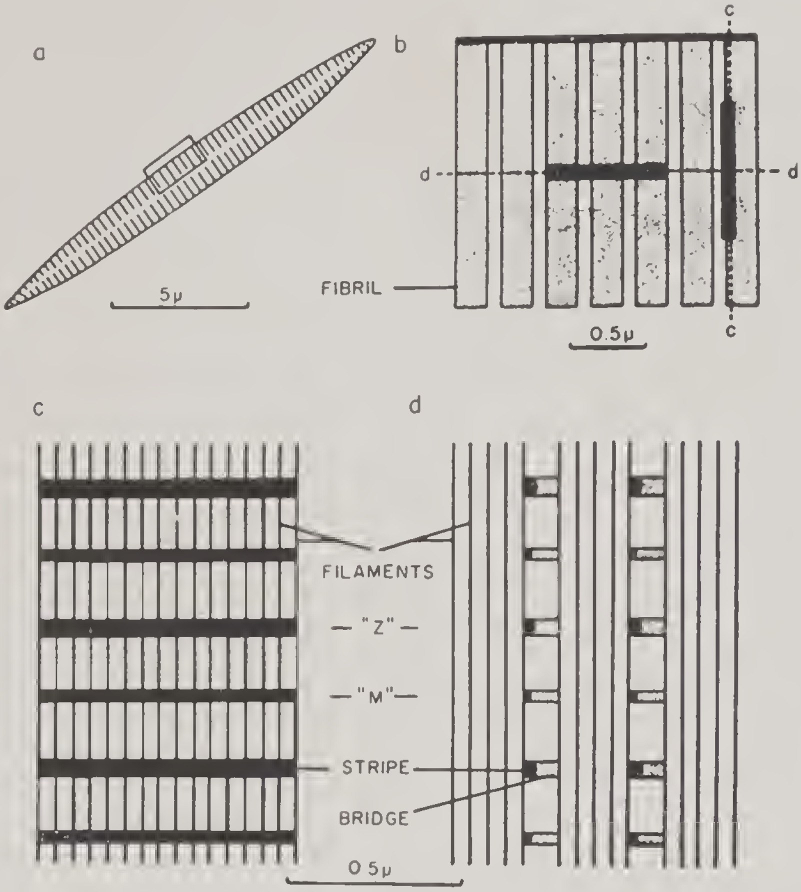

FIG. 6. Diagrams to show the arrangement and fine structure of myofibrils in the cell of the longitudinal muscle coat of the body wall of *Lumbricus terrestris* L. [From Hanson (*36*).] (*a*) Transverse section through the cell. (*b*) An enlarged view of the rectangular region marked in figure 6a showing seven of the myofibrils associated with the cell wall. The black regions (c-c and d-d) indicate the cross sectional areas of the longitudinal sections illustrated in Fig. 3c and 3*d*. (*c*) Stripes and filaments visible in a longitudinal section cut parallel to the faces of the myofibril in a plane represented by c-c. (*d*) Stripes, filaments and bridges visible in a longitudinal section cut perpendicular to the faces of the fibril in a plane represented by d-d.†

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† It is very probable that these so-called "Z" and "M" stripes are concentrations of intermyofibrillar sarcoplasmic reticulum (Hanson, personal communication).

longer side appears to be roughly at right angles to it. The other thin edge of the ribbon-like fibril lies in the interior of the cell close to the corresponding edge of another fibril associated with the opposite surface of the cell (Fig. 6*a*).

A and I bands are not apparent, but superimposed along one surface of the uniform fibril ribbons are regular stripes 300–500 Å wide which Hanson considers to be comparable to the Z and M lines of the striated myofibril.† These stripes appear to be associated with interfibrillar material which binds the fibrils together in the cell. The distance between "Z" lines ranges from 0.5μ in extended muscle to 0.25μ in the contracted state. The fibrils consist of about 100 filaments oriented apparently continuously along the axis, irregularly packed, and about 250 Å in diameter. Between these major filaments which appear homogeneous in cross section, finer filamentous material can be observed.

Usually cross stripes of the type seen in earthworm body-wall muscle are not present in invertebrate smooth muscle. There is considerable variation in arrangement of the filaments; e.g., distinct myofibrils are present in cephalopod smooth muscle but not in that of the snail *Helix pomatia* (*42a*). In the funnel retractor or mantle of the squid and in the pharynx retractor of *Helix pomatia* there is good evidence that the contractile system consists of two types of filaments similar to those present in striated muscle (*48*) (Plate VI). In smooth muscles, however, the double filament system appears to be continuous (*42a*).

III. Relation of Myofibril to Other Intracellular Components

So far as striated skeletal muscle is concerned it seems that the myofibril is practically identical in appearance irrespective of the source of the muscle. Nevertheless, considerable histological differences occur in the striated muscles of a given species and between the same muscle in different species. Two main types of striated muscle cell which are described as the red and white fibers have been distinguished and in the majority of animals, man included, both types of fibers are found in all muscles, the predominating type characterizing the muscle (*49*). In the rabbit, however, muscles usually consist of one of the two types of cell and the red muscles are readily recognized in strong contrast to the more abundant pale muscle tissues.

Many studies have been made on the physiological differences between the two types of muscle [see Needham (*50*) and Ritchie (*8*) for reviews of earlier work] and although the distinctions are not always clear-cut, some generalizations may be made. White muscle is faster, i.e., has a shorter twitch time than red, and although it can undertake short severe bouts of activity it fatigues more rapidly (*8, 50–52*). Red muscle gives a fused contraction at a slower frequency of stimulation. All muscles which contract slowly and repeatedly such as heart and certain postural muscles in vertebrates are red, but on the other hand not all red muscles are slow.

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† See footnote on p. 267.

With certain exceptions (see below) red muscles are capable of sustained activity and their oxygen uptake is greater than that of white muscle (*50, 53*). This high oxidative activity is reflected in the histological appearance. Whereas in white muscles the cell appears packed with myofibrils and mitochondria are few and small (Plate I), in the red fiber the ratio of sarcoplasm to myofibril is high, the myofibrils appear less tightly packed, and large mitochondria are very apparent, e.g., in pigeon breast, heart, and insect flight muscles (*49, 54–57*) (Plate II).

The red color of muscle is due partly to myoglobin, which is probably freely dissolved in the sarcoplasm, and partly to the cytochromes, which are mainly associated with the particulate components of the cytoplasm. Often there is a direct correlation between the myoglobin content of a muscle and its cytochrome level as reflected in the oxidative capacity measured by cytochrome oxidase and succinic oxidase activities (*58, 59*). This general correlation is modified by certain other factors; for example, myoglobin levels appear to be higher in the skeletal muscles of larger animals and it has been suggested that the relatively low myoglobin level of horse heart and pigeon breast muscle compared to the high level of oxidative activity in these tissues is due to their excellent blood supply, which reduces the necessity of a large intracellular oxygen reserve (*58*). In the case of blue whale psoas muscle the myoglobin content is approximately the same as that of horse psoas although the oxidizing enzyme preparations made from the whale are only $\frac{1}{3}$ as active. Thus the oxygen store in whale psoas muscle will last about 20 times as long as that in horse psoas under comparable conditions of activity (*59*) —an important consideration in view of the whale's diving habits. With fishes some correlation is apparent between their activity and the proportion of the muscle which is red (*59a*). It is also reported that the distribution of red muscle is characteristic and can be used for identification purposes in closely related species of fishes (*59a*). The myoglobin level of skeletal muscles can be increased by an extended period of forced exercise (*60*).

In contrast to red muscle, white usually contains relatively large energy-rich phosphate stores and has a high glycolytic capacity. This difference in importance of anaerobic and aerobic metabolism for the two types of striated muscle is further emphasized by studies on the distribution of carnosine and anserine (*61–64*). White muscles such as the pectoral muscle of the fowl, psoas, and longissimus dorsi of rabbit contain large amounts of anserine and little carnosine whereas red muscles such as pigeon pectoral muscle and rabbit semitendinosus contain little of either peptide, and heart none at all (*63*). Davey (*64*) is of the opinion that an inverse correlation with the aerobic capacity of a muscle is more apparent between the dipeptide concentration than the myoglobin content. He considers that the role of the dipeptides is mainly to act as buffers to offset the fall in pH which would result from intensive glycolysis occurring in more anaerobic muscles.

Studies such as those briefly described above suggest that the rate and intensity of mechanical performance of a muscle fiber is to a large degree determined not by the myofibril itself but by the nature of the enzyme systems acting to produce energy-rich phosphate. These enzyme systems are to be found in the sarcoplasm and granular components of the cell: the glycolytic system which is soluble and constitutes the bulk of the sarcoplasmic protein and can produce adenosine triphosphate (ATP) anaerobically in homogeneous phase; and the oxidative phosphorylating system which is localized in the mitochondria. Muscles vary enormously in their dependence upon these two systems in supplying ATP during activity and range from the white muscle of the rabbit which relies mainly on glycolytic processes to the highly aerobic muscles of heart and flight muscles.

Sarcoplasm

Sarcoplasm is usually defined as the soluble cytoplasm in the muscle cell containing for the main part a complex mixture of proteins mostly of the albumin type. All formed elements will be bathed in this protein solution which may be quite concentrated, for phase contrast studies with isolated living frog skeletal muscle suggest that sarcoplasm contains a higher protein concentration than the A band (*39*). Sarcoplasmic extracts are obtained by extracting muscle with low ionic strength buffers which do not bring the myofibrillar proteins in solution. In the past, protein fractions [namely, globulin X (*65*), myogen A (*66*), and myogen B (*66–68*)] have been isolated from sarcoplasmic extracts but subsequent studies have indicated that these fractions are not homogeneous [see Perry (*2a*) for further discussion] and probably do not contain special proteins uniquely characteristic of the muscle cell. The sarcoplasmic proteins, often referred to collectively as the myogens, consist of a large number of different enzymes, in particular those concerned in glycolysis and many others. On electrophoresis these components move in groups of similar mobility and for example at least 11 well-defined gradients can be recognized in rabbit sarcoplasmic extracts

(*69, 70*). The electrophoretic studies on a number of vertebrates (*69–78*) and invertebrates (*79, 80, 80a*) indicate that for skeletal muscle of a given species the pattern is fairly characteristic. Wide variation in electrophoretic pattern occurs between species and between skeletal, cardiac, and smooth muscles. Bosch (*74*) reports that the electrophoretic patterns of sarcoplasm from white and red rabbit skeletal muscle are similar, yet the results of Crepax (*71*) with the same animal certainly indicate some differences in relative sizes of the gradients in extracts from these two types of skeletal muscle.

IV. Protein Components of the Myofibril

The well-defined proteins myosin, actin, and tropomyosin, which have so far been identified as characteristic of muscle tissue, have in the case of rabbit striated muscle, at least, been shown to be exclusively localized in the myofibril.* From this fact and certain unique properties which these proteins possess, it is evident that they must be closely associated with the contractile process and that knowledge of their special properties is of fundamental importance for an understanding of this process.

A. ACTIN

Although myosin has been recognized as the characteristic protein component of muscle tissue since the middle of the 19th century, the other major constituent of the myofibril, actin, is of much more recent origin (*81–83a*). In general actin is less readily extracted from whole muscle than myosin and usually longer extraction times and alkaline pH values are required to bring the former protein into solution. Under such circumstances actin forms the complex actomyosin from which it can not readily be separated, although recently a method for the selective extraction of actin without the preliminary removal of myosin has been published (*84, 85*). The classical procedure which was developed by Straub for the isolation of actin from rabbit muscle involves an initial extraction to remove some of the myosin, followed by a dehydration of the muscle residue with an organic solvent such as acetone. Short periods of extraction of this acetone-dried muscle fiber with distilled water yield clear solutions of low viscosity containing globular or G-actin. On addition of inorganic salts to such solutions a marked increase in viscosity occurs, considered to be the consequence of a polymerization process which converts the actin into the fibrous form (F-actin).

The precise nature of this transformation has yet to be elucidated, but it possesses several unique features which suggest that it may be of significance in the contractile system. For the polymerization of G- to F-actin to take place, the —SH groups of G-actin must remain intact (*86*) and traces of a divalent ion such as Mg or Ca are essential (*87*). Attempts to determine the size of the primary unit of rabbit actin, carried out on the protein depolymerized by reagents such as potassium iodide and the chelating agents ethylenediaminetetraacetate and hexametaphosphate, have yielded results ranging from 70,000–150,000 (*88, 89*). Some light on this divergence of results emerges from Tsao's (*90*) investigations using the polarization-fluorescence technique (*91*). Tsao's results can be interpreted on the basis that G-actin exists in the dimer state (particle weight 140,000) but that in the presence of chelating agents it is dissociated into the monomer form of molecular weight 70,000 by removal of Mg or Ca, which are presumed to bind the two monomer units through their nucleotide prosthetic groups.

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* Hamoir (personal communication) considers that small amounts of tropomyosin may occur in the sarcosomes.

Rather surprisingly Tsao was unable to detect any changes in molecular weight by the polarization-fluorescence method during the polymerization of G- to F-actin, yet electron microscope studies on dilute solutions clearly demonstrate a change in form of the actin micelles from the granular to fibrous form (*92–94*). If the polarization fluorescence results are valid, this implies free rotation about the bonds uniting the actin dimers in F-actin polymer form.

Another feature which is presumably of functional significance is that G-actin preparations invariably contain small amounts of bound nucleotide (*95, 96*), mainly in the form of ATP which becomes dephosphorylated to ADP on transformation [for reviews see Weber (*97*) and Perry (*2a, 98*)]. It is not clear whether this dephosphorylation is in any way related to the increase in isoelectric point which accompanies the formation of F-actin (*99–101*). By dialysis against slightly alkaline media containing traces of ATP and ascorbic acid, F-actin is slowly reconverted to the G-form (*95*).

Those properties of actin which have been described in the literature [for reviews see Weber and Portzehl (*97*), Bailey (*102*), and Perry (*2a*)] refer almost entirely to rabbit actin. In any case, application of the usual physical methods of protein characterization to F-actin are somewhat limited by the extremely thixotropic nature of even dilute solutions of this substance. The ease with which F-actin sediments ($S_{20,w} = 50\text{–}65 \times 10^{-13}$)* makes ultracentrifugation a useful method of purification (*103*) rather than of molecular weight determination.

On treatment of skeletal muscle by the original Straub (*95*) method, solutions of actin can probably be obtained with varying ease from most species. In this way, actin was prepared in similar yields from both femoral and thoracic muscles of the locust, *Locusta migratoria* (*104*), and so far as these investigations were pursued insect actin behaved in a comparable manner to its rabbit counterpart.† More data is available for actin isolated from the cod, *Gadus callarius* L. which closely resembles rabbit actin in characteristic properties, although the specific viscosity of the F-form was somewhat lower (Table II). Adenine compounds were also present in cod G-actin extracts but their significance has not been investigated (*105*). Some data on carp actin has been provided by Hamoir (*72*) who points out that F-actins from the rabbit, cod, and carp all migrate at the same speed during electrophoresis, while cod G-actin apparently moves more slowly than depolymerized rabbit F-actin. This latter observation alone certainly does not necessarily imply significant difference in the two proteins, for the mobility of depolymerized rabbit F-actin can depend on the method of depolymerization (*106*).

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* $S_{20,w}$, sedimentation constant in C.G.S. units reduced to water at 20°.

According to several workers the polymerization of cardiac G-actin possesses certain unusual features. Snellman and Gelotte (*107*, *108*) found such G-actin preparation did not polymerize well and explained this as being due to a deaminase present which acted on the bound actin-nucleotide. In the presence of a cardiac glycoside "Digitotal," which inhibited the deaminase, F-actin obtained from heart resembled that obtained from skeletal muscle. Horvath *et al.* (*109*) have also claimed a specific acceleration of the polymerization of dog cardiac G-actin by low concentrations of g-strophanthin and digitoxin. These authors could demonstrate no effect on the polymerization of G-actin from skeletal muscle even when the g-strophanthin concentration was raised 300–500 times. The relation between these findings and those reported by Snellman and Gelotte is far from clear, and at least one worker does not agree with the view that cardiac glycosides specifically accelerate the polymerization of cardiac G-actin (*110*).

By comparison with rabbit skeletal muscle Cruck (*111*) has identified gradients corresponding to actin in extracts from bovine ventricle, auricle, and bundle of His. Whereas the skeletal muscle actin gradient is very homogeneous, those from the regions of the heart studied were apparently heterogeneous and different from each other. Cruck interprets these findings as indicating that these cardiac tissues contain actins which differ from that of skeletal muscle.

Direct isolation studies on mammalian cardiac muscle and on the skeletal muscle of mammals, fish, and insects indicate the presence of

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† Maruyama (*104a*) reports that actin could not be extracted from acetone-treated tail muscle of the crayfish, *Cambarus clarkii*.

TABLE II

SOME PHYSICAL DATA FOR ACTIN ISOLATED FROM SKELETAL MUSCLE

Animal	Generic name	Muscle	Form of actin	Sedimentation constant (10^{-13})	Diffusion constant (10^{-7} cm.^{2} sec.$^{-1}$)	Molecular weight	Molecular shape
Rabbit	*Lepus cuniculus L.*	Skeletal	G	2.7–4.0[a,b]	2.5[b]	120,000–150,000[b] (dimer)	24 Å × 590 Å (dimer)[c]
Rabbit	*Lepus cuniculus L.*	Skeletal	F	50–65[d]			
Cod[e]	*Gadus callarius L.*	Skeletal	G	3.3 (c = 0)[f]	2.3 (c = 0.3–0.6)	160,000[g] 130,000[h]	Axial ratio 25

[a] Snellman *et al.* (*89*).

[b] Bailey (*102*).

[c] Tsao (*90*).

[d] Weber and Portzehl (*97*).

[e] Connell (*105a*).

[f] c, concentration of protein in grams per 100 ml.

[g] From osmotic pressure measurements.

[h] From sedimentation and diffusion measurements.

actin with similar characteristic properties. These results, together with those obtained in studies on actomyosin extracted from a far wider range of species and types of muscle, make it appear very probable that actin is a universal component of contractile tissue. Further confirmation of its universality is presented by the X-ray diffraction patterns obtained from a wide variety of muscles (*112*) in which the moderate angle reflections are remarkably characteristic. On the basis of comparison with the diffraction patterns given by oriented films of isolated F-actin (*92, 113*), these reflections can be presumed to arise from filaments within the myofibril relatively rich in F-actin (*112*). It is more difficult to generalize about the quantitative distribution of actin in different muscles, particularly as almost the only data to work from consists of estimations of the relative amount of myosin and actomyosin extracted, as obtained from electrophoretic and viscometric determinations. Many factors other than relative abundance can influence the proportions of actin and myosin obtained on simple extraction of muscle tissue. Nevertheless, on the basis of the yields of actomyosin obtained by extraction of the whole tissue, smooth muscle contains considerably less actin than striated muscle, and Csapó (*114, 115*) concludes that the amount increases with increasing physiological activity in the case of uterine muscle.

B. Myosin

1. *Skeletal Muscle*

a. Vertebrate. Almost 100 years ago Kühne used the term myosin to describe the substance which gave rise to a clot when the juice pressed out of frog muscle was allowed to stand at room temperature. Subsequent investigators realized that such extracts were rather complex, and the name was eventually restricted to the globulin which could be extracted from muscle by fairly strong salt solutions. It is probable that most of the myosin preparations described before 1945, and indeed many described since, are in fact actomyosins of low actin content, no special precautions having been taken to remove the latter protein during the preparation. Only since the discovery of actin (*81–83a*) have fairly rigorous criteria been applied to establish the purity of myosin preparations. Although myosin has now been prepared from striated muscle of a wide range of species, most of the detailed physicochemical studies have been restricted to the protein isolated from rabbit skeletal muscle. For details of these investigations a number of recent reviews should be consulted (*2a, 97, 102, 116–118a*).

As rabbit myosin is fairly well characterized, it is frequently used as the reference protein in comparative studies and therefore its main physicochemical characteristics will be briefly presented here. Authors have used varying terminology to indicate whether their preparations contain actin or not. In accordance with Weber (*97*) L-myosin will be used to designate actin-free myosin which is probably identical with Dubuisson's β-myosin (*70*), Szent-Györgyi's "crystalline" myosin (*119*), and myosin T, the name proposed by Bailey (*102*) for Tsao's actin-free preparation (*120*). Myosin A (*81*) is an actin-poor preparation of myosin, whereas myosin B (*81*) is an actomyosin made by direct extraction of muscle and contains relatively large amounts of actin. Likewise, Dubuisson's electrophoretic component, α-myosin (*70*), is an actomyosin. Following Bailey's suggestion (*102*), myosin preparations described before the discovery of actin or those described more recently, which by their method of preparation very likely contain actin, will be denoted as (acto-) myosin.

Rabbit L-myosin forms clear viscous solutions in the presence of neutral salts (e.g., $\geqslant 0.3\ M$ KC1) and exhibits the characteristic globulin property of precipitating when the ionic strength is lowered. If the myosin gel is washed free of salts, it swells due to the Donnan effect, finally passing into a pseudosol state and for this reason it has sometimes been described as water soluble (*119*). All physical measurements indicate that the molecule is extremely asymmetric, estimates of its length ranging from 2300 Å (*97*) to 1500 Å (*121*). Until recently, the molecular weight of L-myosin has been taken as 840,000, based on the sedimentation-diffusion and osmotic studies (*97*). It is possible that such a value represents the weight of a stepwise aggregate of myosin, for several workers (*122, 123, 123a*) have obtained values for the sedimentation constant which are lower than that reported by Weber and Portzehl; the figures have been particularly low when measurements were carried out at 4° on myosin which was not exposed to temperatures greater than 1° during its preparation. Under similar conditions the diffusion coefficient is also higher than the figure obtained by Weber and Portzehl. Some evidence is available which suggests that aggregation can occur when myosin is left standing at room temperature for a few hours (*123, 123a*). Nevertheless Portzehl (*124*) reports 840,000 for the molecular weight obtained by osmometry with a myosin preparation maintained at 0° throughout its preparation. Laki and Carroll (*123*) deduce a molecular weight of 500,000 from their results and it seems possible that the true minimum molecular weight for myosin is 420,000—a value which would agree well with that suggested by studies on the meromyosins (see below).

Rabbit myosin possesses the interesting property of being split by trypsin (*125–131*) and chymotrypsin (*132*) into two characteristic subunits which share between them the properties of the myosin molecule. These are L-meromyosin (molecular weight 96,000) and H-meromyosin (molecular weight 232,000), the latter possessing the actin-combining and the ATPase properties. The myosin molecule is considered to contain the L- and H-meromyosin subunits in the ratio of 2 to 1 (*129*); consequently the minimum molecular weight for myosin on this basis would be 424,000. High concentrations of urea depolymerize L-meromyosin into units of 4600 molecular weight, called protomyosins, which are uniform in size and shape but differ in amino acid composition (*132a*). As yet there are to the author's knowledge no published accounts of the meromyosins derived from other myosins.* Such a study might be of great interest in studying the phylogenetic interrelations of the myosins.

There is as yet no evidence for qualitative differences between the myosin of red and white skeletal muscle in a given animal. Crepax (*71*) in electrophoretic studies on various striated muscles of the rabbit found that although both α-myosin (actomyosin) and β-myosin (L-myosin) gradients were apparent in extracts obtained at $\mu = 0.35$, $pH = 7.1$, from both types of muscle, rather less of these proteins was extracted from the white muscle. Crepax also noted that actomyosin was formed more rapidly in extracts from red muscle and in extracts from rabbit myocardium than was the case in similar extracts from white muscle. Cardiac extracts contained the α- and β-myosin gradients with mobilities characteristic of similar components isolated from skeletal muscle. On the other hand, although components could be extracted from rabbit foetal muscle which appeared similar to α- and β-myosins, their electrophoretic mobilities differed distinctly from those of the adult animal (*71*).

A detailed study by Miller *et al.* (*133*) on myosins extracted from the hind-leg muscle of rabbit, rat and mouse emphasizes how closely these mammalian proteins resemble one another. Extracts of whole muscle purified by a single precipitation were used and all contained α-, β-, and γ-myosins. The preparations were remarkably similar in yield, electrophoretic mobility, sedimentation rate, specific refractive increment, and viscosity, although some difference in stability of the solutions was noted.

Of the cold-blooded vertebrates so far studied, the turtle, *Testudo graeca*, like the mammals, readily gives extracts in which the α- and β-myosin peaks can be recognized. In the case of teleostean fishes, however, it is not possible to demonstrate the presence of the β-myosin peak under similar conditions (*72, 134*). As considerable difficulty exists in the preparation of actin-free fish myosin, there is a shortage of the data required for adequate comparison of the mammalian and fish proteins.

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* Needham and Cawkwell (*132b*) have prepared by tryptic digestion of uterine actomyosin a fraction which sediments after 2 hours at 100,000 g, represents 48% of the protein, and possesses most of the original ATPase activity.

Hamoir (*73, 135, 136*) confirmed an earlier observation of Roth (*72*) that neutral extracts of fish muscle invariably contained actomyosin rather than myosin, and has shown further that if extracts of carp muscle are made at pH 6.5, $\mu = 1.0$,† a component sedimenting with the same mobility as β-myosin of rabbit can be recognized. On ultracentrifugation this component gave rise to 2 peaks of similar sedimentation rate (*72*). Despite this apparent heterogeneity of the β-myosin peak of carp muscle extracts, Hamoir (*73, 136*) has established the existence of a carp β-myosin with the characteristic sedimentation velocity by ultracentrifugation of carp actomyosin in the presence of ATP. Connell (*105, 105a*) has prepared a myosin from cod, *Gadus callarius* L., which although similar to rabbit myosin in many of its properties, has a much greater tendency to aggregate than rabbit myosin.

Skeletal muscle of the frog, *Rana temporaria*, resembles that of the teleosts in that extracts of the tissue contain α-myosin but no β-myosin (*76*). By electrophoresis of such extracts in the presence of ATP, a myosin gradient is revealed which unlike the β-myosin of the rat, mouse, turtle, carp, cod, and lobster has an electrophoretic mobility not identical to but greater than that of rabbit myosin (*137*). Strangely enough, frog myosin produced by treating actomyosin with ATP sediments at a rate comparable to that of rabbit myosin (*137*). It seems reasonable to explain the absence of the β-myosin from neutral salt extracts of the striated muscle from a number of vertebrates as being due to the rapid breakdown of ATP (or perhaps to the low initial level of ATP) in these extracts compared to similar extracts from the rabbit and other warm-blooded animals (*73, 137*).

b. Invertebrate. Striated muscle is widespread in the arthropods and among insects in particular, but due no doubt to the problem of getting sufficient tissue in the first instance physicochemical studies on insect myosin are extremely rare. For the most part, those studies which have been carried out on insect muscle proteins have been concerned with ATPase or apyrase activities. Although well-characterized insect L-myosin has not yet been isolated, Gilmour and Calaby (*104*) have studied actomyosin, purified by four precipitations, isolated from two species of the locust, *Locusta migratoria* L. and *Gastrimargus musicus* (Fabr.). The indirect flight muscles of these insects are from a morphological point of view more or less intermediate between normal skeletal muscle and the "fibrillar" flight muscles of Diptera and Hyme-

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† Ionic strength, $\mu = \frac{1}{2}\Sigma cz^2$

TABLE III

PHYSICAL DATA FOR L-MYOSIN ISOLATED FROM SKELETAL, CARDIAC, AND SMOOTH MUSCLES

Animal	Generic name	Muscle	Sedimentation constant (10^{-13})	Diffusion constant (10^{-7} cm.^{2} sec^{-1})	Molecular weight	Molecular shape
Rabbit	*Lepus cuniculus L.*	Skeletal[a]	7.1 ($c = 0$)	0.87 ($c = 0$)	850,000	23 Å × 2300 Å
		Skeletal[b]	6.15 ($c = 0, 4°$)	1.0 ($c = 0, 1°$)	500,000	
		Skeletal[c]	5.3 ($c = 0.36, 25°$)			
Rat		Skeletal[d]	6.2 ($c = 0, 7°$)			
Mouse		Skeletal[d]	6.2 ($c = 0, 7°$)			
Cod	*Gadus callarius*	Skeletal[e]	6.45 ($c = 0$)	1.10 ($c = 0.19 - 0.40$)	530,000	Axial ratio 60–70
Carp		Skeletal[f]	6.55 ($c = 0$)			
Dog		Heart[g]	3.5–4.9		100,000	Frictional ratio 1.2
Sheep		Heart[h]	6.5–7.2 ($c = 0$)			
Man		Uterus[i]	5			
	Pecten maximus	Adductor[j]	6.4 ($c = 0.15$)[k]			

[a] Weber and Portzehl (*97*). [b] Laki and Carroll (*123*). [c] Holtzer (*123a*). [d] Miller *et al.* (*133*). [e] Connell (*105a*). [f] Hamoir (*72*). [g] Ellenbogen and Olson (*142*). [h] Gelotte (*140*). [i] Csapó *et al.* (148). [j] Rüegg (*156*). [k] The value for the sedimentation constant refers to the main component observed in this preparation.

noptera, whereas the femoral muscles of the locust which were also used in this study are comparable in appearance to most vertebrate skeletal muscles. Short periods of extraction with alkaline KCl solutions (Weber-Edsall) produced actomyosin solutions, and prolonged extraction of either kind of muscle did not increase the actin content. Extracts from the femoral muscle were usually more viscous and of the myosin B-type, whereas the relatively more fragile thoracic muscle was resistent to extraction and extracts were intermediate in properties between myosin A and B. An increase in viscosity on the addition of rabbit or insect actin to thoracic extracts suggested the presence of uncombined myosin. Hodge (*29*) also noted that the proteins of isolated fresh and glycerated myofibrils of the blowfly (*Lucilia cuprina*) flight muscle were much more resistant to extraction than rabbit skeletal myofibrils under comparable conditions.

Little work has been carried out on crustacean myosins with the exception of that of the lobster. Certain amino acid analyses have been carried out by Bailey (*138*) on (acto-) myosin. Electrophoretic studies on extracts of whole abdominal muscle of this invertebrate are reported by Dubuisson-Brouha (*80*), who demonstrated the presence of α- and β-myosin peaks in extracts carried out at $\mu = 0.35$, pH 7.5. Although myosin in general could be extracted from lobster at lower ionic strength than is the case with rabbit muscle, in the ease with which β-myosin could be detected the lobster extracts resembled those from the rabbit rather than those of most other cold-blooded invertebrates.

In summarizing the results of comparative studies on the myosins from the skeletal muscles of a wide range of species, both vertebrate and invertebrate, it is apparent that there is a lack of the quantitative physicochemical data which are essential to base sound conclusions. Most of the evidence is of an electrophoretic nature, yet it suggests a remarkable homogeneity in the nature of β-myosin wherever it is found. Anomalies do exist but these may be due in some cases to peculiarities in the stability of the myosin isolated from certain species. Differences are observed in the relative proportions of α- and β-myosin which appear in extracts of whole muscle; these differences do not necessarily indicate differences in the β-myosin itself, but rather possible differences in its state of association with actin *in situ*. More likely they are a reflection of the ATP level of such extracts and their efficiency in maintaining the level of this nucleotide at the expense of glycolysis.

Complete amino acid analyses of myosin by modern analytical methods have been performed on rabbit myosin, this being the only myosin which has so far been obtained in a state of purity which merits such study (*139*). Earlier Bailey (*138*) analyzed (acto-) myosins of low actin content for total N, amide N, total S, cystine, methionine, tyrosine, and tryptophan. For the 6 different myosins studied, the mammalian and avian figures were remarkably close, and only comparatively small deviations were recorded in the case of fish and lobster myosins. Like the physicochemical and enzymatic investigations, these analytical studies suggest some fundamental plan in the structure and composition of the myosins isolated from voluntary striated muscle.

2. *Cardiac muscle*

Few studies have been reported on cardiac myosin and the fragmentary quantitative data available do not allow us to generalize very widely on the nature of cardiac myosin in relation to its skeletal equivalent. Myosins prepared from sheep, calf, cow, and pig hearts resemble one another closely and are comparable to rabbit skeletal myosin in electrophoretic mobility and sedimentation constant (see Table III) although the value of the latter shows some irregular variation with concentration (*140*). ATPase studies have been described on rabbit and pig (acto-) myosin (*141*) bovine, sheep, pig (*140*), and dog cardiac myosins (*142*). It appears that the ATPase activity of cardiac myosin is about 10–20% that of rabbit skeletal myosin measured under identical conditions and that the enzyme is less stable (*140*). Dog cardiac myosin possesses a comparatively low sedimentation constant, and a molecular weight of 100,000 which is appreciably smaller than has yet been obtained for other myosins of any origin (*142*). Crepax (*71*) noted that both α- and β-myosin gradients were apparent in extracts of myocardium from rabbit and horse although the amounts extracted were less than obtained from rabbit skeletal muscle and also α-myosin (actomyosin) was relatively more abundant. As in studies on other tissues, this latter fact could be due to the low ATP level of mycardium extracts. There is some evidence that the relative contractile protein content of different regions of the heart depends on their function (*111*, *143*). On the basis of viscometric studies of extracts made on rabbit and pig hearts, Kovats (*143*) concludes that more myosin can be extracted from the ventricles than from the less active auricles.

3. *Smooth muscle*

a. Vertebrate. Most investigations concerned with the contractile proteins of vertebrate smooth muscle have been confined to the uterus although an (acto-) myosin-like protein from beef intestine (*144*) and actomyosin isolated from swine esophagus muscle (*144a*) have been described. Recently Crepax (*71*) has examined the extracts of bovine bladder muscle by electrophoretic methods. Yields obtained using the conventional methods of myosin and actomyosin preparation are very much lower than those obtained from skeletal muscle (*71, 145, 146*). The presence of actomyosin in uterine extracts is indicated by a reversible drop in viscosity on the addition of ATP, albeit the drop is rather low. In general, although actomyosin-like protein can be obtained from uterus, the pattern of the electrophoretic diagrams of extracts made at high ionic strength differs markedly from such extracts of striated muscle (*71*). Csapó (*147*) has studied in detail the viscosity changes occurring on addition of ATP to uterine actomyosin.

Although uterine myosin has not yet been purified, it has been identified as the slower moving ($S_{20,w} \sim 5$) of the two polydisperse components observed on ultracentrifugation of actomyosin extracts from human uterus (*148*). The amount of rapidly sedimenting material ($S_{20} > 20$) identified as actomyosin was greater in uteri of pregnant women compared to those from nonpregnant women. In further studies carried out on actomyosin preparations from uteri of humans, rats, and cows, Csapó (*114, 148*) concludes on the basis of viscometric studies that uterine muscle contains less myosin and actomyosin than striated muscle and that the percentage of actomyosin of the total myosin plus actomyosin is less. During pregnancy, the total amounts of these two components and the percentage contribution of the actomyosin increase, reaching a maximum immediately before parturition (*115*). The ATPase activity of uterine actomyosin preparations, studied by a viscometric method, is appreciably less than either cardiac or striated muscle preparations assayed in a similar way, but the effects of Mg and Ca on the activity suggest that it is of the myosin type (*147*). Such a conclusion is confirmed by the more detailed studies of Needham and Cawkwell (*146*) who prepared an actomyosin-like protein from pig and rabbit myometrium and from the whole uterus of the rat. They demonstrated specific ATPase activity, greater activation by Ca than Mg, and stimulation by 2, 4-dinitrophenol. Although some anomalies exist in the effects of KC1 on the enzymatic behavior of uterine actomyosin compared to the rabbit skeletal enzyme, the properties of the two ATPases are very similar.

b. Invertebrate. During their classical work on the physicochemical characterization of vertebrate (acto-) myosins, Von Muralt and Edsall (*149*) produced evidence that a similar protein, solutions of which possessed streaming birefringence, could be extracted from the foot muscle of the marine snail, *Busycon*. Mehl (*150*) extended these findings by determining the molecular lengths of (acto-) myosins from the smooth muscle of the octupus, *Polypus bimaculatus*, the snail, *Polynices lewisii*, which he compared with the results of similar studies on the striated muscle of the sting ray, *Urobatis halleri,* and the rabbit. Although unable to extract myosin in these investigations from the sea anenome, *Cribrina xantho-grammica,* or the sea cucumber, *Stichopus californicus,* he concluded that the difference between mollusk and vertebrate (acto-) myosins was no greater than that existing between two species of mollusks. In the case of the sea anenome at least, Mehl's results are not due to the absence of actomyosin from the smooth muscle of this organism, for more recently Maruyama (*151*) has reported the extraction of an actomyosin-like protein with apyrase activity from *Anthopleura japonica.* It is possible that Mehl did not break up the cells sufficiently to allow extraction of the contractile proteins to occur.

Electrophoretic studies strengthen the case for the similarity between the contractile protein system of invertebrate smooth muscle and that of vertebrate striated tissue; in extracts of the foot muscle of the mollusk, *Murex brandaris* (*152*), and the body-wall muscle of the worm, *Lumbricus terrestris* L. (*80a*), α- and β-myosins can be recognized moving with the mobilities characteristic of these components as isolated from rabbit. According to the Dubuisson school, one of the few differences existing between the myosin extracted from vertebrate striated muscle compared with those from invertebrate smooth and striated muscle, lies in the fact that the latter are extracted at lower ionic strengths. It is questionable, however, whether this phenomenon can explain the anomalous behavior of the squid, *Ommastrephes sloani pacificus,* reported by Migita and Matsumoto (*153*). The authors found that on extraction of the mantle muscle with distilled water the second and third extracts showed pronounced streaming birefringence and appreciable amounts of the total nitrogen passed into solution. This phenomenon was also observed with the muscles of other mollusks but not with those of teleosts and is probably due, as the authors themselves hint, to some peculiarity of molluscan myosin or actomyosin which causes its dispersion from intact muscle under salt-free conditions in a manner similar to the solution of previously extracted L-myosin of rabbit in the complete absence of salt.

Only recently have detailed studies of molluscan myosin been attempted. Tonomura *et al.* (*154, 155*) have found that the ATP sensitivity of myosin B from the fast adductor of *Pecten yessoensis* is greater than similar preparations from the slow adductor. These authors claim that arginine appears to play a specific role in reformation of actomyosin after dissociation by ATP (see Section IV,C). By employing salt fractionation procedures, these authors have shown that myosin B preparations from both adductors contain actomyosin and myosin. Although the myosin B from the fast adductor is 3 times as active as an ATPase than

Myosin B from the slow adductor, Tonomura *et al.* conclude that activities for unit functional weight, i.e., the weight of protein bound with 1 gm. molecule ATP, were almost identical. The only convincing preparation to date of an L-myosin from molluscan smooth muscle has been reported by Rüegg (*156*) from *Pecten maximus*. This was obtained by ultracentrifugation of purified actomyosin dissociated by the addition of ATP and magnesium (*157*). *Pecten* myosin was electrophoretically homogeneous and $S_{20,w} = 6.7$.

C. The Interaction of Actin and Myosin

Unless special precautions are taken, extraction of whole muscle or isolated myofibrils by media of higher ionic strength gives solutions of myosin containing varying amounts of actin. The viscosity of such (acto-) myosin extracts was shown by Szent-Györgyi and collaborators (*81, 83a, 119*) and by the Needhams and their associates (*158, 159*) to fall on the addition of low concentrations of ATP. When the ATP was hydrolyzed the viscosity returned to the original value. This viscosity change was originally interpreted by Szent-Györgyi as being due to the dissociation by ATP of the complex formed between F-actin and myosin, which he called actomyosin, into its component proteins. On hydrolysis of the ATP to ADP, the proteins were considered to combine once again to reform the complex, which has a viscosity considerably higher than the sum of the viscosities of the two components taken separately. It has been claimed on the basis of light-scattering studies that the particle weight of actomyosin does not decrease in the presence of ATP (*160*). Careful re-examination of the effect with the same technique (*161*) and with the ultracentrifuge (*157*) confirms that dissociation into actin and myosin does indeed take place, although Tonomura (*161a*) considers the amount of dissociation to be small.*

The interaction of the myofibrillar proteins actin and myosin is obviously of fundamental importance in the conversion of chemical into mechanical energy because ATP, which itself undergoes enzymatic change, profoundly modifies this interaction and induces physical changes in the system. The precise nature of the physical changes depends upon the ionic environment and whether relaxing factors (see Section VIII) are present or not.

Actomyosin extracted as such from muscle is often referred to as "natural" actomyosin to distinguish it from so-called "synthetic" actomyosin prepared by combining samples of actin and myosin which have been separately isolated and purified. Actomyosin systems commonly used for study of these mechano-chemical effects are as follows and the effects of ATP described are those occurring in the absence of relaxing factors.

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* Recent studies on the flow birefrigence of myosin B solutions support the view that ATP dissociates the actomyosin complex into actin and myosin (*161b*).

1. *Extracted Actomyosin Systems*

a. Sol state. At $\mu > 0.3$, pH 7.0, actomyosin forms a viscous solution and addition of ATP brings about a reversible fall in viscosity.

b. Gel state. At $\mu \sim 0.05$, pH 7.0 actomyosin precipitates and in the presence of low concentrations of Mg salts responds to ATP by a marked synaeresis, the water content of the gel falling from 97–98% to ~50%. When this occurs in suspension the particles of actomyosin decrease in size and increase in density, and rapidly settle—a phenomenon described as superprecipitation. If the actomyosin is precipitated by extrusion of the sol into a solution of low ionic strength, a thread is formed which shortens on the application of ATP. Such threads are not very strongly oriented and develop rather weak tensions when treated with ATP, but some improvement can be obtained by increasing the orientation and the protein concentration.

2. *Unextracted Actomyosin Systems*

Initial extraction of actomyosin destroys the orientation of these proteins, and once extracted it is very difficult to obtain actomyosin gels which contain anything like the protein concentration in the myofibril, i.e., 15–20%. Unextracted actomyosin systems containing actin and myosin oriented as *in situ* are as follows:

a. Isolated Myofibrils. Myofibrils washed free of sarcoplasm respond to the application of ATP by pronounced shortening. In suspensions a change in opacity occurs and the ATP contracted myofibrils settle rapidly. The change resembles in some respects the superprecipitation phenomenon observed with actomyosin suspensions.

b. Glycerated Fibers. These are prepared by storing thin strips of muscle, held at resting length, for several days in 50% glycerol at —10° (*162*). This procedure extracts most of the endogenous ATP and part of the sarcoplasmic proteins leaving the myofibrillar structures apparently intact. Muscles built up of long parallel fibers, such as the rabbit psoas are particularly suitable for such preparations. Thin fiber bundles teased out from such glycerated strips contract violently in the presence of ATP under suitable ionic conditions and can be conveniently used to study tension development under isotonic and isometric conditions. The maximum tension developed by glycerated fiber models (0.5 kg./cm.^{2}–5 kg./cm.^{2}) approximates to that developed by the living muscle from which it was prepared (*163*).

3. *Properties and Comparative Aspects of Actomyosin Systems*

Complexes with actomyosin-like properties have been extracted from all the muscles so far studied (see Section IV,B) although considerable variation exists in the intensity of the response obtained. Likewise myofibrils which contract on the addition of ATP have been isolated from a number of tissues, including skeletal muscle of the rabbit (*98, 164*), pigeon, rat, domestic hen (*165*), the blowfly *Lucilia cuprina* (*29*), the housefly *Musca* (*29*), the beetle *Dytiscus* (*166*), and the body muscle of the earthworm *Lumbricus terrestris* (*36*). There is little doubt that the contraction induced by ATP is a universal property of myofibrils irrespective of their origin.

Studies have been made in glycerated fibers prepared from a number of muscles both striated and smooth, for example, rabbit psoas (*97, 119, 162, 163, 167*) sartorius, rectus, anconaeus, and cutaneos pectoris muscles of the frog (*168, 169*), muscle from *Limulus* (*119, 170*), posterior adductor and retractor pedis of *Anodonta* and the longitudinal fibers of cow's rectum (*171*). The maximum tension exerted by glycerated fibers of rabbit psoas when treated with ATP is approximately the same as that reported for the yellow adductor of *Anodonta,* although in the former case the tension is limited by the tensile strength of the fiber model. Lower maximum tensions were recorded with cow rectum and frog skeletal muscle fiber models (*163, 172*).

In addition to ATP, the triphosphates of uridine, guanosine, cytidine, and inosine all dissociate actomyosin sols and induce contraction or superprecipitation in the gels (*173–175*). Inorganic polyphosphates such as pyrophosphate (*176, 177*) and tripolyphosphate (*159, 178, 179*) dissociate actomyosin sols, but these substances have a plasticizing rather than a contractile action on the gel systems (see Section VIII). Under certain conditions tripolyphosphate has been reported to have slight contracting activity on glycerated fibers (*179*).

Little is known about the detailed mechanism of the interaction between actin and myosin. Its relative insensitivity to ionic strength indicates that it is not of the simple electrostatic type. Intact myosin —SH groups are essential (*97, 163, 180–182*) but the more reactive F-actin —SH groups appear not to be required for the interaction (*182*), although the —SH groups of G-actin must be intact for polymerization to F-actin to take place (*183–185*). The interaction of actin and myosin of insects appears to involve sulfhydryl groups as does the rabbit system upon which most of the work has been done. In his study of the contraction and superprecipitation of actomyosin gels prepared from 6 species of insects, Maruyama (*186*) was able to demonstrate inhibition of this process by low concentrations of copper sulfate in the case of preparations from the thoracic muscle of two species of the dragonfly, *Orthetrum albistrum* and *Sympertrum frequense.*

The interaction is absolutely specific for actin and myosin but it does not appear to be species specific. For example, rabbit actin will form actomyosin with locust myosin (*104*) and actomyosin has also been formed with the components isolated from different species in a number of other cases (*187*). Although myosin from the langouste interacts with vertebrate myosin, it has been reported that actin from this invertebrate does not form actomyosin with vertebrate myosin (*187*). Information is not available as to whether this is due to the difficulty of preparing active langouste actin or whether a real anomaly exists in the case of this protein. After tryptic conversion of myosin into H-meromyosin the ability to combine with actin is retained although superprecipitation no longer takes place (*130, 131*).

On the basis of studies on the purities of myosins from uterine, skeletal, and cardiac muscles it has been concluded that the firmness with which actin is bound to myosin decreases with increasing physiological activity of the muscle (*108*). This view receives some support from work on locust thoracic and femoral muscles (*104*), yet the binding in swine esophagus actomyosin is claimed to be weaker than in its skeletal counterpart (*144a*). This latter finding is perhaps more compatible with the report that for a given weight of myosin B the number of ATP combining sites is larger for striated muscle than the slower smooth muscles (*144a*).

There is some evidence that additional factors may be concerned in the interaction of actin and myosin isolated from smooth muscle. For example, Csapó (*147*) finds that a soluble, heat-labile, nondialyzable factor is required to increase the rate of viscosity rise after treatment of vertebrate uterine actomyosin with ATP. Such extracts also restored contractility to actomyosin threads which had lost this ability through storage. It is not possible to say what is the relation between the factor described by Csapó and the requirement for arginine and a heat labile factor which Tonomura *et al.* (*154, 155*) have found to play a part in the restoration of the viscosity of actomyosin preparations from adductors of *Pecten yessoensis* after ATP addition. Although indispensable for the above effect, arginine was not required for the superprecipitation of *Pecten* myosin B.

The interaction of actin myosin and ATP, in addition to producing the physical changes described above, also involves the enzymatic hydrolysis of ATP (see Section VI). Actin is not essential for this enzymatic change but its presence may profoundly modify its characteristics. As yet no detailed mechanism for the interaction can be given and indeed there are suggestions that the simple concept of dissociation of actomyosin into its components may be an oversimplification. Tsao's (*188*) results with the polarization-fluorescence technique can be interpreted to mean that combination of myosin and F-actin to give actomyosin involves the formation of the monomer form of actin, molecular weight 70,000, the polymerized dimer form which constitutes F-actin being reformed on the addition of ATP. The assessment of the significance of these latter results must await a final decision on the validity of the polarization-fluorescence method.

D. Tropomyosin

1. *Characteristics and Distribution*

Tropomyosin, the protein first isolated by Bailey (*189, 190*) in 1946 from rabbit skeletal muscle, is the only component of the contractile unit which has been subjected to any determined comparative study. Such investigations have been facilitated by the fact that tropomyosin possesses certain characteristic properties and can be crystallized in the conventional sense. Tropomyosin from rabbit skeletal muscle is characterized by: (*a*) the formation of highly polymerized viscous solutions at neutral pH values in the absence of salt; (*b*) reversible depolymerization by salts; (*c*) molecular asymmetry exceeding 20; (*d*) relative ease of crystallization; (*e*) resistance to the denaturing effect of organic solvents and acid pH values; (*f*) the absence of tryptophan and proline from its amino acid make-up, and the presence of quite small amounts of glycine, phenylalanine, histidine, and cystine and very large amounts of dicarboxylic acid and total base; (*g*) the absence of N-terminal amino groups as disclosed by the fluorodinitrobenzene method. These features are in general retained by tropomyosins isolated from striated and smooth muscles of a wide range of vertebrate and invertebrate phyla.

In the original Bailey (*190*) procedure, tropomyosin is precipitated in the range 40–70% saturated ammonium sulfate. Employing methods identical to or based on this method, Tsao and collaborators (*191–194*) have isolated tropomyosin and nucleotropomyosin (see below) from the striated and smooth muscles of a number of different species, both vertebrate and invertebrate. These results are summarized in Table IV. For the striated muscles analyzed, the amounts of tropomyosin are rather similar to the figure originally obtained by Bailey from rabbit muscle, i.e., 0.5 gm. per 100 gm. wet weight. It is very probable that the figures obtained by the classical procedures are low, for recent investigations (*106*) show that on treatment of isolated rabbit skeletal myofibrils by slightly alkaline buffers of low ionic strength the tropomyosin extracted represented at least 1.5 gm. per 100 gm. wet weight of whole muscle. With the exception of preparations from pigeon breast and snake skeletal muscle, Sheng and Tsao (*191*) were able to achieve crystallization of the protein as plates or needles often very similar to rabbit tropomyosin. According to Tsao's group the tropomyosin content of pig heart is lower than that of most striated muscles whereas the smooth muscle of duck gizzard contains 2–5 times as much. Nevertheless the latter value is still below the highest value obtained for rabbit skeletal muscle (*106*). Although on a wet weight basis the tropomyosin values for bovine uterus are low, Sheng and Tsao (*191*) consider that they represent about 10% of the structural proteins of this tissue. They could find little change in the nucleotropomyosin content of uterus before and during the various stages of pregnancy. Likewise there is little change in tropomyosin content of bovine embryonic muscle during development when it is calculated on a dry weight basis (*191*).

Fish tropomyosins have received some attention. Bailey (*190*) originally isolated this protein from whiting, and Hamoir has carried out detailed studies on carp tropomyosin (*72, 195–197*).

With the possible exception of prawn tropomyosin which salted out in the range 15–30% saturated ammonium sulfate, the proteins described by Tsao and collaborators are all precipitated in the range 40–70% saturated ammonium sulfate. Recently Bailey (*44, 45*) has described a tropomyosin from both smooth and striated muscle of the pseudolamellibranch, *Pinna nobilis,* which precipitates out on dialysis against 0.2 *M* KCl and salts out at 23–29% ammonium sulfate. A similar protein, slightly more soluble than that obtained from *Pinna,* can be isolated from arm muscle of the cephalopod *Octopus vulgaris* (*45*) and the smooth adductor of *Pecten maximus* (*156*). In addition to these insoluble tropomyosins, Bailey had reported the existence of a water-soluble tropomyosin present in low concentration in extracts from both *Pinna* and octopus muscle. After removal of the "insoluble" tropomyosin from extracts of octopus muscle, the soluble tropomyosin can be crystallized in small rosettes of needles (*197a*). By dialysis of such mother liquors under conditions similar to those recommended for rabbit tropomyosin (*190*), Rüegg has also prepared plate-like crystals from the adductor muscle of the lamellibranch, *Pecten maximus.* The tropomyosin isolated by Yoshimura (*198*) from muscle of the squid, *Ommastrephes sloani pacificus* Steenstrup, is probably of the soluble kind since the method employed was based on that described by Bailey for rabbit muscle.

TABLE IV
ANALYTICAL AND PHYSICOCHEMICAL DATA FOR THE TROPOMYOSINS

Animal	Muscle	Amount present			Sedimentation constant (10^{-13})	Diffusion constant (10^{-7}cm.$^{-2}$ sec.$^{-1}$)	Molecular weight	Molecular shape (a/b)	N-Terminal residues	C-Terminal residues	Crystal form
		% Wet weight	% Dry weight	% Myofibrillar protein							
Rabbit	Skeletal	0.5^a		$\sim 12^b$	$2.6\ (c=0.6)^c$	$2.4\ (c=0.6)^c$	$53{,}000^e$	29^e	Nil^f	1 isoleucine^d	Plates
	Skeletal				$2.95\ (c=0)^d$	$\sim 4.5\ (c=0)^d$	$59{,}000^d$			1 serine	
Pig	Cardiac	$0.21^{a,g}$	1.16^g	4.2^g			$89{,}000^h$	36^h	Nil^i		
Rat	Skeletal	$0.21^{i,j}$									
Carp	Skeletal	$0.40^{j,g}$			2.7^j						
Pigeon	Breast	0.59^g									
Toad	Skeletal	0.41^g									
Prawn	Skeletal						$74{,}000\text{-}80{,}000^h$	22^h	Nil^i		Needles
Snake		0.55^g									
Crab		0.18^g									Needles
Beef	Embryonic	$0.09-0.17^g$	1.23^g	10.5^g							
	Gravid uterus			11.1^g						1 Leucine**^{d} 1 Asparagine	Fine needles
	Uterus				$2.95\ (c=0)^d$						
	Bladder				$2.95\ (c=0)^d$	$\sim 4.5\ (c=0)^d$				1 Isoleucine^d 1 Serine^d	Coarse needles

Duck	Gizzard	1.1[g]					147,000–133,000[h]	50[h]	Nil[i]		
Sepia	Mantle						68,000[h]	55[h]	Nil(?)[i]		Needles
Squid (*Ommastrephes sloani pacificus* Steenstrup)		0.38[k]					54,000[k]				
Pinna nobilis	Adductor		30*[l]		3.1 ($c = 0$)*[m]	2.21 ($c = 0$)*[m]	135,000*[m]	1400 Å × 18.5 Å*[m]			Needles
Oyster	Adductor		25*[l]								
Scallop (*Pecten maximus*)	Smooth adductor				3.0 ($c = 0$)[n]						Needles
	Smooth adductor		30*[o]								*Needles
	Striated adductor				3.0 ($c = 0$)[n]	2.3 ($c = 0.22$)[n]	~ 100,000[n]				Plates
	Striated adductor		<5*[n]								*Needles

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* These data refer to insoluble tropomyosin (globulin tropomyosin, tropomyosin A). All other data refer to soluble tropomyosin (tropomyosin B).

** Human uterine tropomyosin.

[a] Bailey (*190*). [b] Perry and Corsi (*106*). [c] Bailey (*102*). [d] Kominz *et al.* (*47*). [e] Tsao *et al.* (194*a*). [f] Bailey (*194b*). [g] Sheng and Tsao (*191*). The data from this reference refer to nucleotropomyosins. [h] Tsao *et al.* (*192*). [i] Jen and Taso (*194*). [j] Hamoir (*73*). [k] Yoshimura (*198*). [l] Bailey (*44*). [m] Kay (*204*). [n] Rüegg (*204a*). [o] Rüegg (*156*).

Kominz and associates (*46, 47*) have also isolated two types of tropomyosin from the clam, *Venus mercenaria*. The most abundant of the two, which precipitated in the range 28–40% saturated ammonium sulfate, they called tropomyosin A; the other type, which precipitated in the range 45–65% saturated ammonium sulfate and was present in smaller amounts, they designated as tropomyosin B. Tropomyosins A and B are presumably identical to Bailey's insoluble and soluble forms isolated from *Pinna nobilis* and *Octopus vulgaris*.

Laki and collaborators (*46, 47*) have examined tropomyosins (precipitated in the range 40–70% saturated ammonium sulfate) from cardiac, skeletal, and smooth muscle of mammals, from unspecified muscles of lower vertebrates (amphibia, fish, and cyclostome) and from various invertebrates (arthropod, mollusk, echinoderm, annelid, and nemertean). Most of these proteins crystallize in plates or various types of needles and all have sedimentation constants falling along the same line and extrapolating to an $S_{20,w}(c = 0)$ of about 3.0 (Fig. 7).

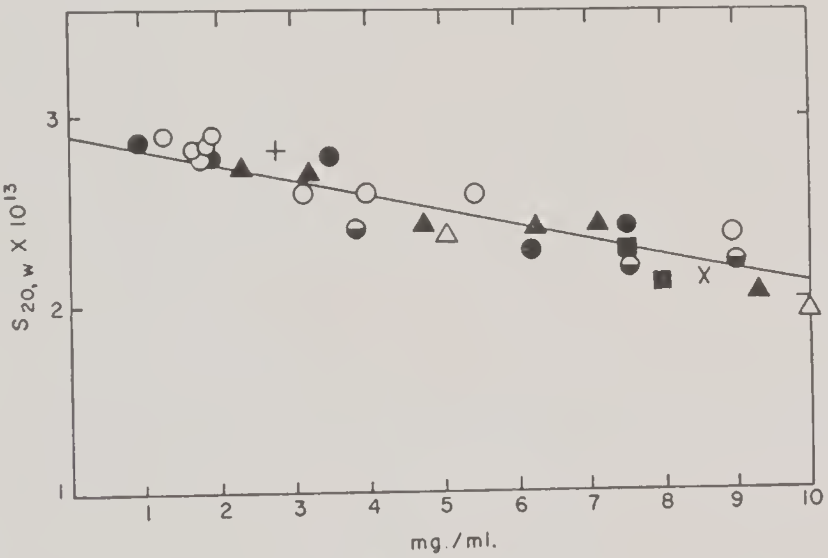

FIG. 7. Sedimentation of tropomyosins prepared from striated and smooth muscles. KEY: ▲, rabbit skeletal muscle; ●, bovine bladder; ■, rabbit uterus; △, flatworm; ×, earthworm; +, lobster; ◒, Venus foot; ○, rabbit skeletal L-meromyosin.

From the amino acid analyses of Laki and collaborators and of Bailey and Yoshimura, certain generalizations can be made which emphasize the fundamental similarity of the tropomyosins of water soluble and water insoluble types isolated from a wide range of species. The glutamic content is high (160–200/10^5 gm.), proline is absent or of very low content, and histidine is low (*45–47, 198*). The sum of the basic groups is always about 140/10^5 gm. and in the lower phyla arginine tends to replace lysine. Also in the lower phyla, the decrease in dicarboxylic acid content and an increase in amide ammonia results in a lower net anionic charge. Although the amino acid pattern of the tropomyosins has definite characteristics, within the comparatively few analyses available exceptions to these trends do exist; many more analyses from various species within the phyla will have to be made before we can say whether these variations in amino acid composition are of real significance (see Saad *et al. 198a*).

In summary it may be stated that all types of muscle contain protein which can be classified as of the tropomyosin type in so far as most of the general criteria listed earlier are satisfied. From the studies carried out so far the tropomyosins appear to fall into two main groups distinguished by their solubility properties. Although the nomenclature is somewhat confusing these are the so-called soluble tropomyosins (tropomyosin B) and the insoluble tropomyosins (globulin tropomyosin or tropomyosin A).

The soluble type of tropomyosin has been isolated from the striated and smooth muscle of vertebrates and invertebrates. On the other hand the insoluble tropomyosin has only been obtained from invertebrate smooth muscle where it is present in large amounts, particularly in molluscan adductor muscle. In consequence a very large proportion of the total protein of the latter muscles is of the tropomyosin type.

2. *Nucleotropomyosin*

Tropomyosin as originally isolated by Bailey is free from nucleic acid, but in 1951 Hamoir (*195, 196*) isolated from carp skeletal muscle tropomyosin comparable to Bailey's preparation from rabbit, both in the free form and as a complex with nucleic acid. This complex could be crystallized, contained up to 10–20% nucleic acid usually of the pentose type, and was electrophoretically indistinguishable from the nucleic-acid-free protein. Tsao and Sheng (*191*) have reported the isolation of nucleotropomyosin from a wide range of species with nucleic acid content, always of the pentose type, ranging from 0.7% in duck gizzard to 16–17% in nongravid bovine uterus, heart, and crab skeletal muscle. It is uncertain whether tropomyosin exists *in situ* as a ribonucleoprotein complex but it seems probable that, once extracted, under suitable conditions of ionic strength and pH tropomyosin can form a complex with pentose nucleic acid from various structures within the muscle cell.* Such a conclusion is based on the following facts. (*a*) Variable amounts of nucleic acid are found in nucleotropomyosin isolated from a given source. (*b*) Rabbit myofibrils contain low amounts of nucleic acid and even this may be due to contamination with endoplasmic reticular structures themselves rich in nucleic acid. (*c*) The amount of nucleic acid in pig heart nucleotropomyosin increases with the degree of comminution of the tissue (*191*). (*d*) The nucleic acid and protein components are loosely associated as they move independently on electrophoresis (*193, 195*).

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* It is probable that the ribonucleic acid present in nucleotropomyosin arises from contamination by the ribonucleoprotein which can be isolated independently from the myofibril. See Section IV,E,2.

3. *Role of Tropomyosin*

Tropomyosin has been isolated only from muscle tissue and this fact, together with the knowledge that it is localized in the myofibril suggests that it either has a contractile role or is an essential structural component of the contractile system. In view of the properties of this protein and certain features of its amino acid composition, Bailey suggested that tropomyosin may be one of the ultimate units from which myosin is composed (*190*). The slightly higher tropomyosin content of the muscles of young animals and fetal muscle gives some support to this idea. More recently this view has been stated in a somewhat more positive fashion by Kominz *et al.* (*139*) as a result of their amino acid analyses of the muscle proteins. A mixture of equal proportions of tropomyosin and actin approximates the amino acid composition of myosin although the agreement with some of the amino acids is poor.

The comparative studies of Sheng and Tsao (*191*) indicate that in general the tropomyosin content of smooth muscle is higher than that of striated muscle from the same species. Taken on face value this could indicate a relation between tropomyosin content and function, but it cannot at this stage be decided whether the differences reflect differences in the ease with which tropomyosin can be extracted rather than absolute differences in content. Nevertheless strong support for a relation between function and tropomyosin content is provided by the smooth adductor muscle of *Pinna nobilis* and the oyster where the tropomyosin of the insoluble type constitutes 30% and 25% respectively of the total protein. One feature possessed by smooth muscle from *Pinna* and other mollusks is their slow rate of relaxation (*9*) and it is tempting to suppose as Bailey (*45*) has done that tropomyosin plays a part in this "holding" function. For example in *Pinna* the fully active state lasts only 200 milliseconds whereas the tension takes about 12 seconds to decay completely (*9*). It is interesting to note that the speed of contraction of molluscan smooth muscle is not very different in some cases (e.g., the gray part of *Pinna* adductor) from some striated vertebrate muscles. Sheng and Tsao (*191*) have also commented on the possibility of tropomyosin playing a role in the special function of smooth muscle in holding at either the contracted or extended state.

The smooth portion of *Pecten* adductor muscle consists of two distinct parts of different opacity (*198b*). The less opaque portion contains roughly equal amounts of actomyosin and tropomyosin and possesses ATPase activity 5 times as great as that of the more opaque portion. In the latter part of the smooth adductor only 15–25% of the total globulin is actomyosin, the remainder being tropomyosin. Significantly, with glycerated fiber models which have been contracted isometrically the rate of stress relaxation after quick release is much slower in the tropomyosin rich portion, while resistance to stretch is higher (*198b*). Some attempt has been made to explain the behavior of living smooth adductor muscles of a variety of lamellibranch mollusks on the basis that tension is developed during the active state by the formation of linkages in a sliding-filament actomyosin system. The phenomenon of passive tension which is a feature of these muscles could then be due to the formation with rigid paramyosin filaments of other linkages which break at a very slow rate (*198c*).

4. *Paramyosin*

Paramyosin is the name given to a component occurring in the foot and adductor muscle of the clam, *Venus mercenaria* and other molluscan muscles (neck and adductors of *Mya arenaria;* adductors of *Mytilis edulis, Ostrea virginica, Pecten magellenica;* foot of *Viviparis* sp.), which gives rise to the characteristic X-ray diffraction pattern first described in *Venus* by Bear (*199*). Subsequent electron optical studies (*200*) showed that the fine needle-like fibrils which could be isolated from *Venus* adductor muscle stained in a complex and regular fashion (see Fig. 8) to give rise to a periodic cross striation of 145 Å. With remarkable agreement, Bear found the meridional spots in his study of dried strips of the muscle to be all orders of 145 Å and concluded that the repeating fiber axis period is $5 \times 145 = 725$ Å. In a more recent study, Bear and Selby (*201*) point out that the paramyosin (type I) pattern is characteristic of the white portion of *Venus* adductor, whereas the tinted portion gives the type II pattern which is typical of actin (*92, 113*).

Paramyosin fibrils are insoluble at low ionic strength ($< 0.3\ M$ KCl) in the range pH 5–7, but begin to pass into solution in $0.6\ M$ KCl and at lower pH values (*200, 202*). Hodge (*202*) found that the dialysis against water of dilute acetic acid extracts of paramyosin fibrils precipitated the protein as a translucent gel of fine filaments; but if the dialysis was carried out at pH 3.5 $\mu = 0.35$, filaments with a characteristic striation of repeat period 1400 were observed. At $\mu \geqslant 0.1$ the solution exhibited one electrophoretic component, and in the ultracentrifuge

80% of the material sedimented as a homogeneous peak, with a smaller peak sedimenting more rapidly. At lower ionic strengths both electrophoretic and sedimentation gradients became asymmetric and the diffusion constant increased by a factor of 10, findings which are consistent with dissociation of a complex into a multiplicity of components rather than a simple depolymerization. Hodge (*202*) also reported that paramyosin fibrils were the only periodically striated structures which could be observed in preparations from *Venus*, and more recently observations on cross sections of molluscan adductor ("catch") muscles suggest that the bulk of the total protein is situated in the fibrils (*42a, 202a*). A large proportion of the paramyosin fibrils consists of "insoluble tropomyosin." Bailey (*45*) suggested in view of the amounts present and some preliminary X-ray diffraction studies by Beighton (unpublished observations) that insoluble tropomyosin and paramyosin were identical. This view has been substantiated by the preparation from paramyosin fibrils of crystals of "insoluble" tropomyosin possessing the same banded structure as the original fibrils when fixed and stained in the same manner (*203*) (Fig. 8). Several different transverse band patterns are observed in tropomyosin needles. The simplest pattern repeats every 145 Å, some every 725 Å, and further variation of the simple patterns are obtained with regular differences in density of the bands. Kay (*204*) has proposed on the basis of light-scattering, sedimentation-diffusion, and viscosity measurements that the dimensions of the protein molecule are 1400 × 19 Å. Thus the length is twice the paramyosin spacing (725 Å) and equal to the 1400 Å band structure observed by Hodge (*202*) in precipitated protein filaments from extracts of paramyosin fibrils.

Actomyosin and myosin have been isolated from pecten adductors (*154–156*), myosin ATPase activity being greater in the fast (tinted) portion (*204a*). Although paramyosin fibrils have been reported as possessing weak, Ca-activated ATPase activity (*204b*), it has not yet been unequivocally shown whether the actomyosin system is localized in the paramyosin fibrils or in other structures in the cell. Certainly other proteins would appear to be present, as the yield of insoluble tropomyosin from adductors is not great enough to account for all the protein presumed to be present in paramyosin fibrils.

It is of interest that the two characteristic X-ray diffraction patterns observed in muscle are the type I (paramyosin) and the type II (actin). The former is characteristic of white portion of molluscan adductors whereas the latter is found in striated muscle and the tinted or gray part of molluscan adductors. In both cases the protein responsible must be oriented, presumably in fibrils, along the axis of the muscle. Also it

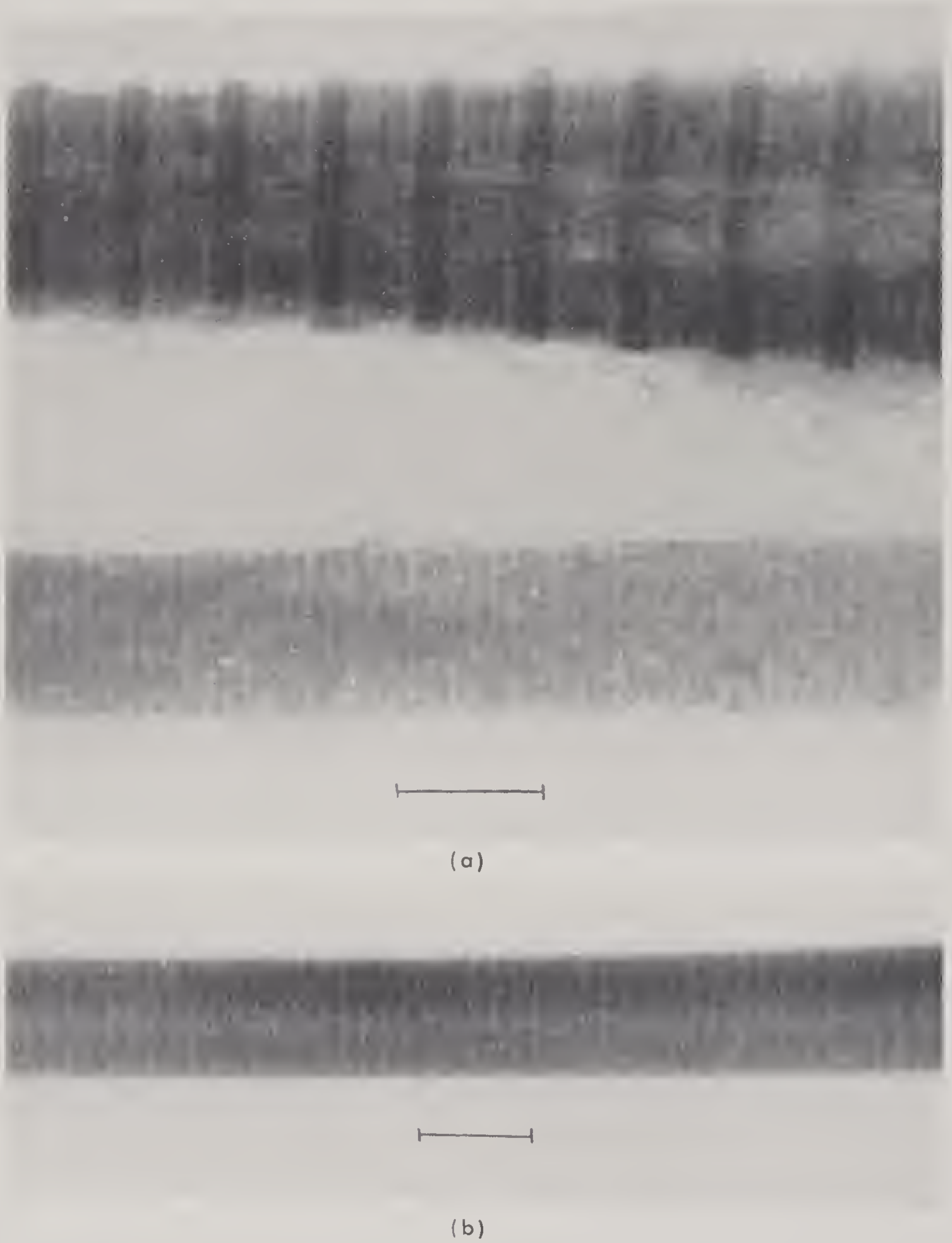

Fig. 8. (*a*) Electron micrograph of two crystals of water insoluble tropomyosin from the white adductor muscle of the Portuguese oyster. (*b*) Electron micrograph of a paramyosin filament isolated from the same source. Magnification marks, 0.1 μ. [From Hanson *et al.* (*203*).]

appears that these two proteins are present in complementary amounts, e.g., smooth muscles rich in tropomyosin contain low amounts of actin and vice versa in striated muscle. There are indications also from studies on vertebrate striated myofibrils that soluble tropomyosin and actin are associated *in situ* (*106*). Other evidence which can be interpreted to indicate a special association of these two proteins *in situ* is provided by the report of the isolation of a complex of nucleotropomyosin, actin, and a "phosphate-absorbing" protein from human and bovine uteri (*145*). Bearing in mind the scanty evidence available, it is possible that one of the fundamental filamentous systems of the myofibril is composed of actin and tropomyosin associated together in a way which determines the functional characteristics of the muscle. On the one hand in skeletal and cardiac muscles, which are capable of a rapid build-up and decay of tension, actin is relatively more abundant than tropomyosin. On the other hand, when tension decays slowly as in smooth muscle, exemplified in the extreme by bivalve slow adductor muscle, tropomyosin-type proteins dominate this filamentous system.

E. Other Myofibrillar Proteins

During the last decade evidence has been presented for the existence of a number of myofibrillar proteins other than actin, myosin, and tropomyosin. Often these additional components have been isolated from extracts of whole muscle or found to be associated with one of the recognized myofibrillar proteins and it is frequently difficult to decide if they are constituents of the myofibril *in situ*. Sarcoplasm probably contains several hundred different enzymes the last traces of which are difficult to remove during the isolation of myosin, actin, and tropomyosin. More definite studies are possible on isolated myofibrils but even after repeated washing these structures contain proteins which resemble those of the sarcoplasm in certain of their properties (*165*). Quantitatively the amounts of proteins other than myosin, actin, and tropomyosin must be comparatively small, for these three components make up about 90% of the total myofibril. It might be supposed that the various cross lines such as the Z band, M line, etc. consist of protein not as yet characterized, and likewise small amounts of unidentified proteins may be present within the myofilaments themselves.

1. *Y-Protein*

Electrophoresis of extracts of normal, resting rabbit muscle made with KCl solution ($\mu > 0.5$) reveals a component travelling slower than β-myosin at $\mu = 0.4$ and pH = 7.4 (*205*). Dubuisson considers this gradient to be due to a component which he calls the Y-protein. Electrophoresis of purified Y-protein gives a single peak slightly asymmetric, although in the ultracentrifuge the appearance of three main peaks suggests the preparation is polydisperse (*206*). Y-protein possesses globulin properties in that it is precipitated by 35% saturated ammonium sulfate and is insoluble in distilled water and fairly soluble at $\mu = 0.005$.

Like β-myosin, Y-protein cannot be extracted from muscle previously subjected to contracture or from muscle in rigor mortis and Dubuisson considers that this observation indicates that Y-protein is part of the contractile system. Once extracted Y-protein is fairly soluble, but the fact that solutions of high ionic strength are required to extract the protein from muscle, suggests that it is in some way bound to the insoluble structural part of the cell rather than being in the sarcoplasm.

The gradient corresponding to the Y-protein has also been observed in skeletal muscle from the frog (*76*), the earthworm *Lumbricus terrestris* (*80a*), and from the carp (*77*). A gradient of similar mobility has been reported in extracts of rabbit cardiac muscle (*71*) but is absent in the turtle *Testudo graeca* (*77*) and the lobster (*77, 80*). A gradient (Z) which has a higher mobility than both myosin and the Y-protein is found in extracts from these latter two animals in which the Y-gradient is absent. The Z-gradient resembles the Y-gradient in that it only appears in high ionic strength extracts from relaxed muscle.

2. *Extra Protein or X-Protein*

According to Szent-Györgyi *et al.* (*207*), when myosin is selectively extracted from glycerated myofibrils a fraction representing on average ~18% of the total myofibrillar N is also extracted. This N fraction may not be entirely protein since part is reported as passing through dialysis membranes. Such a figure for the extra protein, or X-protein, as it has been called (*208, 209*), seems rather high for protein material which is not myosin, actin, or tropomyosin. Hanson and Huxley (*208, 209*) found this fraction represented 11% of the total myofibrillar protein, whereas Perry and Corsi (*106*) estimated the nondialyzable N of this fraction to be 3–7% of the total N of well-washed myofibrils from both fresh and glycerated rabbit muscle. Recent electrophoretic (*210*) and chromatographic (*211*) analyses of this fraction reveals that it contains 3–5 components one of which has been identified as tropomyosin. Villafranca (*212*) has isolated a homogeneous protein component which he considers to make up the bulk of the extra protein fraction from rabbit skeletal muscle. This protein shows remarkable similarity to tropomyosin in that it is reversibly depolymerized by salt but apparently has a higher molecular weight and precipitates at lower ammonium sulfate concentrations.

It seems probable that the protein isolated by Villafranca is a complex of tropomyosin and other components which are present in the extra protein fraction. A globulin fraction which is insoluble in distilled water has been isolated from extra protein fraction (*211*). This protein moves as one main electrophoretic component but is apparently less homogeneous on ultracentrifugation (*211*). Also from the extra protein fraction a ribonucleoprotein has been isolated (*211a*) which is associated in some way with isolated myofibrils and which is probably the contaminant responsible for the ribonucleic acid found associated with myosin and certain tropomyosin preparations.

3. *Contractin, γ-Myosin*

Electrophoretic diagrams of high ionic strength extracts of contracted or contractured muscle differ from those made from resting muscle in that the β-myosin gradient disappears and another gradient moving slower than β-myosin is apparent (*70, 213, 214*) (see Fig. 9).

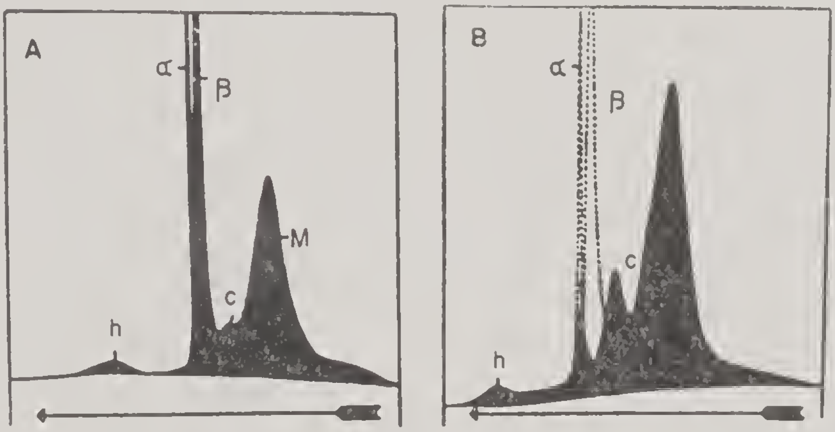

FIG. 9. Electrophoretic diagrams of extracts of rabbit skeletal muscle containing sarcoplasmic and myofibrillar proteins. [After Dubuisson (*70*).] Conditions: $\mu = 0.35$, pH 7.4, 50,000 seconds. KEY: A, normal resting muscle (obtained by slow cooling of the tissue); B, contracted muscle (obtained by stimulation and immobilization in liquid air when maximally contracted); α, actomyosin; β, myosin; M, myogens; C, contractin; h, myoalbumen.

This latter new gradient has been ascribed by Dubuisson to a protein which he calls contractin. The amount of contractin appears to bear a definite relation to the degree of contraction of the muscle and has been found in larger amounts in molluscan foot muscles (*70*). Contractin has a similar electrophoretic mobility to γ-myosin (*152*), the slow electrophoretic component present in small quantities in Edsall-Weber myosin preparations, and Dubuisson (*70*) suggests that these two proteins may be identical. Although γ-myosin has not yet been precisely defined it has been further studied by Azzone (*214a*) and shown to complex with myosin.

4. *Metamyosin*

In preparations of L-myosin obtained by the method of Mommaerts and Parrish (*215*), Raeber *et al.* (*216*) have observed in addition to contractin, a slower electrophoretic component which they call metamyosin. This protein has been obtained in an electrophoretically homogeneous state but there is no evidence to suggest that it is localized in the myofibril other than its association with purified myosin preparations. It is claimed to represent 5–20% of myosin preparations and has been obtained from rabbit skeletal and sheep embryonic muscle.

On electrophoresis metamyosin moves as two main components and contains nucleic acid and 5′-adenylic acid deaminase as contaminants (*216a*). Metamyosin appears to be a complex of several components of the extra protein (cf. reference *211*).

5. *Delta-Protein*

Amberson and collaborators (*217–219*) have isolated from extracts of whole rabbit muscle a water-soluble protein fraction (Delta-protein) which moves as a fast electrophoretic component. Taking into account the fact that it is extracted from muscle by high ionic strength solutions in addition to its fibrous nature and its property of complexing with myosin, Amberson *et al.* imply that Delta-protein is a component of the contractile system. It is clear both from electrophoretic and ultracentrifuge studies that Delta-protein is heterogeneous.

6. *Component C and Other Components*

Electrophoresis of extracts from isolated, well-washed rabbit myofibrils reveal, in addition to tropomyosin and an inactive form of actin, a third slow component representing 1–2% of the total myofibrillar protein (*106*). The amounts present can be reduced by repeated washing of the myofibrils, and it cannot yet be decided whether this protein represents a true myofibrillar component or a sarcoplasmic fraction associated with the myofibril and not readily removed (*106*). This fraction has been called component C (*196*). Certainly chromatographic analysis of various extracts of repeatedly washed myofibril preparations yields small fractions comparable in behavior to the sarcoplasmic proteins. Evidence does not yet allow us to decide if these are real myofibrillar components or sarcoplasmic contaminants.

Tsao (*219a,b*) has also reported the isolation of minor protein components of the myofibril which do not appear to be identical with other structural proteins reported in the literature.

V. Localization of Myofibrillar Components

A. PROTEINS

Almost without exception all the analytical data available is related to the myofibril of rabbit skeletal muscle, and although this tissue is probably representative of moderately active vertebrate skeletal muscle there are suggestions that quantitative variations exist in other types of striated muscle. Certainly there are appreciable differences in the amounts of the fibrillar proteins present in smooth compared to rabbit skeletal muscle.

The isolated rabbit myofibril contains at least 90–95% protein together with small amounts of lipid, nucleic acid, and inorganic material (*2a*). It is not certain whether the two former constituents are integral components or merely adventitious contaminants arising during the preparation—possibly from filaments of sarcoplasmic reticulum attached or adhering to the myofibril. Completely satisfactory analytical data for the amounts of the different proteins in the myofibril are not yet available. This is because considerable difficulty exists in analyzing mixtures of muscle proteins due to their physical properties and their readiness to interact, and due to the fact that the myofibril probably contains as yet uncharacterized components. From analyses carried out on whole muscle by Hasselbach and Schneider (*220*) it is possible to estimate the composition of the myofibril if the contribution of the myofibrillar protein to the total muscle protein is known. It is probable however that some error arises from the fact that part of the stroma fraction also contains myofibrillar protein. Hanson and Huxley (*208, 209*) have attempted to reconstruct the myofibril in terms of its proteins by optical measurements with the interference microscope and biochemical analysis of isolated glycerated myofibrils. Many studies suffer from the fact that little account has been taken of tropomyosin content, and that few efforts have been made to characterize the fractions extracted. This latter fact is particularly important with proteins such as actin, myosin, and tropomyosin which so readily interact and coprecipitate. The analytical figures in Table V combine the results of the investigations already mentioned and those of Perry and Corsi (*106, 221–223*) and in the view of the author represent the closest approximation to the myofibrillar composition which can be made to date.

TABLE V

APPROXIMATE PROTEIN COMPOSITION OF THE ISOLATED RABBIT MYOFIBRIL

Component	Per cent
Myosin	50–55
Actin	20–25
Tropomyosin	10–15
Other components	5–10

Vital for an understanding of the mechanism of contraction in striated muscle is a knowledge of how the myofibrillar proteins are distributed within the sarcomere. It is apparent that there is an uneven distribution of protein along the sarcomere length but, apart from suggestions that there was a concentration of myosin in the A band based largely on the fact that myosin solutions themselves were strongly birefringent (*224, 225*), it is only recently that direct information on this aspect has become available. There is now good evidence that probably all the myosin is concentrated on the A band. This view is based on the following types of evidence: (*a*) Selective extraction of myosin from whole muscle (*226*) and myofibrils isolated from glycerated muscle (*16, 17*) causes the disappearance of the A band and a marked fall in birefringence of this band. These findings, supported by the results of combined biochemical and interference microscope studies, can be interpreted to imply that at least ⅘ of the myosin is present in the A band (*208, 209*).* (*b*) Studies with fluorescent antibodies specific for myosin indicate a concentration of this protein in the A band (*227*). (*c*) On extraction of myofibrils from fresh muscle under conditions of low ionic strength when actin and tropomyosin pass into solution but myosin does not, the Z band and the I band disappear (*106, 223*).

Selective extraction experiments do not allow us to decide finally whether the myosin is evenly distributed in the A band of the myofibril at rest length, i.e., with the H band apparent. From the scanty evidence available there are suggestions that it is not evenly distributed, or at least that there are differences in association with actin and tropomyosin in different regions (*223*). After exhaustive extraction of the myofibril under conditions when actin, tropomyosin, and small amounts of an unidentified fraction, component C, pass into solution—these representing altogether 35–40% of the total protein—the surviving A bands appear denser at the edges (*223*).

Actin and tropomyosin are best considered together because, although there is no evidence definitely suggesting independent localization, there is circumstantial evidence of *in situ* association and suggestion of a complementary structural function. For example, on extraction of inactive actin and tropomyosin from the myofibril under varying conditions the relative proportions of the two proteins in the extract remain constant (*106*), although from consideration of the solubility properties of the extracted proteins this would not necessarily be expected. It is significant that the characteristic features of the X-ray diffraction pattern of muscle is that of actin in the striated muscles of invertebrates and vertebrates, whereas in molluscan smooth muscle it is replaced by the diffraction pattern characteristic of paramyosin, for whose identity with the tropomyosin proteins there is good evidence. This implies that either protein can be the main component of filamentous structures aligned along the axis of the cell.

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* Extraction of isolated myofibrils of the horseshoe crab, *Limulus polyphemus*, with solutions which extract mainly myosin in the case of the rabbit, removed the A band but yielded actomyosin (*226a*).

The evidence of direct extraction experiments (*106, 223*) indicates that the I band proper must consist of actin tropomyosin and possibly small amounts of unidentified protein. It also follows from the fact that myosin is concentrated in the A band that actin, the next most abundant component, must be present in the I band. Extraction experiments indicate the presence of both actin (*17, 106, 223*) and tropomyosin (*106, 223*) in the A band but there is some controversy as to whether or not the actin occurs in the H space. Implicit in the model of Hanson and Huxley (*17*) is the view that actin filaments do not extend across the H space. The biochemical evidence that invoked these authors to support this view is that after extraction with pyrophosphate solution, which they presume to remove myosin free from actin, a gap appears in the center of the sarcomere which is considered to correspond to the H space. This evidence can only be used to support the view that actin is absent from the H space if the extracts are shown to be free from actin. On later admission of Hanson and Huxley (*209*) such extracts can contain actin as other workers have demonstrated (*106, 223*). Hasselbach (*226*) has reported continuity of the residual filaments of the myofibril after selective extraction of myosin, and the extraction of actin and tropomyosin from isolated myofibrils from fresh muscle brings about a widening and fall in density of the H space (*106, 223*).

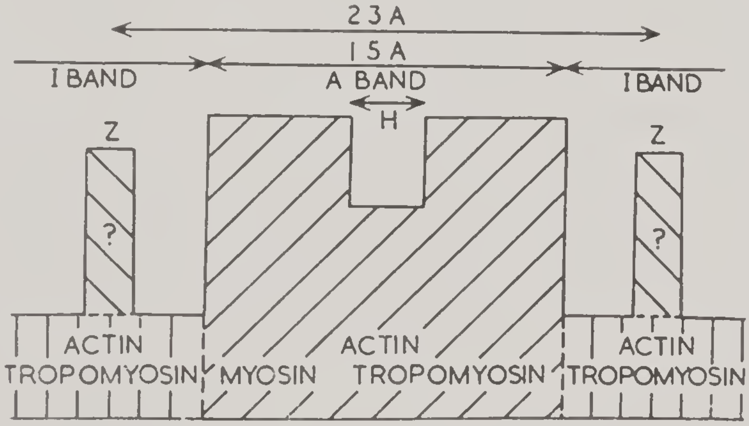

FIG. 10. Diagram illustrating the distribution of proteins in the rabbit skeletal myofibril at rest length. Vertical dimensions indicate the relative protein densities at different points along the myofibril as indicated by interference microscope studies (*208*).

Nothing is known about the composition of the Z band although it has been estimated from studies with the interference microscope to represent about 6% of the total myofibril protein (*208*). On the basis of the above interpretation of the experimental evidence, a schematic representation of the distribution of the proteins in the rabbit skeletal myofibril is given in Fig. 10.

B. NUCLEOTIDES

Apart from the proteins already described above, the only other significant component which has so far been shown to be present in the isolated nucleotide is the so-called bound nucleotide (*98*). This component consists of adenosine phosphates, 70–80% of which is adenosine diphosphate in the case of well-washed myofibrils from rabbit skeletal muscle. The nucleotide represents 90–140 μg. of acid-labile phosphorus per gram of myofibrils isolated from the cardiac and skeletal muscle of the rabbit and skeletal muscle of the rat. Similar values have been obtained for frog gastrocnemius, claw muscle of the river crab *Astacus astacus,* skeletal muscle of the stag beetle *Lucanus cervus,* smooth muscle from the crop of the hen, foot of the river mussel *Unio pictorum,* and the body-wall of the earthworm *Lumbricus terrestris* (*228*).

It must be presumed that this nucleotide is bound to the actin in the myofibril. The fact of its occurrence in muscle tissues of widely differing types, taken together with the fact that it is found in relatively constant amounts, strongly suggests some important function in the myofibril (*222*). Systems for phosphorylating ADP are widespread in cells but the myofibrillar nucleotide, representing in the case of rabbit skeletal muscle about 10% of the total adenosine found as polyphosphate in this tissue, is unavailable to myokinase and creatine phosphokinase (*222, 229*). As yet it is not possible to say whether *in vivo* the myofibrillar nucleotide is fully phosphorylated at any stage of the contraction-relaxation cycle, nor has phosphorylation so far been demonstrated *in vitro.*†

Present evidence indicates that actin is to be found in both I and A bands and it might be expected that there is a similar distribution of the bound nucleotides. The only data available on the myofibrillar distribution of adenine nucleotides, however, point to a concentration in the I band in resting muscle (*230, 231*). It does not follow, however, as has been concluded on the basis of these measurements of absorption at 257 mμ, that there is a concentration of ATP in the I band of resting muscle. Absorption in the ultraviolet would also be observed if myofibrillar nucleic acid was concentrated in this band or if the band was overlaid by nucleic-acid-rich structures such as granules or endoplasmic reticulum. In frog skeletal muscle a significantly higher concentration of radioactive ATP accumulates in the region of the boundary between the A and I bands when the isolated muscle is soaked in a solution containing radioactive adenine (*231a*).

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† Although evidence has been presented (*229a*) for the phosphorylation of the bound ADP of F-actin during depolymerization to G-actin in the presence of the creatinephosphokinase system, it appears that *in situ* the phosphate part of the myofibrillar nucleotide only slowly equilibrates with the nucleotide pool within the cell (*229b*). Exercise did not appreciably speed up this interchange suggesting that reversible polymerization of actin does not occur during muscle contraction.

C. Enzymatic Activities of the Myofibril

The only enzyme as yet shown to be indisputably associated with the structural elements of the myofibril is myosin adenosine triphosphatase [(ATPase); see Section VI,A]. No recognized enzymatic activity has been found in pure actin or tropomyosin preparations.

Rabbit myofibrils and myosin preparations possess marked 5′-adenylic deaminase activity but present evidence indicates that this enzyme is not part of the myosin molecule but in some muscles at least it is essentially a sarcoplasmic component (see Perry, *2a*, for review). In the author's laboratory, Newton (*232*) has shown that L-myosin and myofibrils from pigeon breast muscle can be readily prepared free of 5-adenylic deaminase activity despite the fact that this tissue is rich in the enzyme. Whereas in cell fractionation studies with pigeon breast muscle the 5′-adenylic deaminase is found practically entirely in the sarcoplasmic fraction, in rabbit psoas muscle the total activity is divided roughly equally between the sarcoplasm and the myofibrils.

Recently it has been claimed on spectrophotometric evidence that deamination of adenine compounds occurs during a single twitch (*233*) although this is not borne out by Mommaerts' (*234*) analysis of the chemical changes associated with this event. Certainly deamination of adenylic acid does not appear to be essential for contraction as 5′-adenylic deaminase is present in mammalian stomach and heart muscles and in crab claw muscles (*235*) in low concentrations and is absent from skeletal muscle of the locust (*104*) and the crayfish (*235a*) and from the fast adductor of *Pecten yessoensis* (*235b*). A specific deaminase for ADP has been demonstrated in rabbit actomyosin (*236*) and myofibrils (*237*) although the capacity of these structures to deaminate ADP is only a few per cent of their adenylic acid deaminating power (*2a*).

Oxidizing enzyme systems have not been demonstrated in myofibrils (*2a, 55*) but traces of myokinase, aldolase, and other sarcoplasmic enzymes are usually present in well-washed preparations.

VI. Adenosine Triphosphatases of Muscle

Since the discovery that most of the ATPase activity of skeletal muscle is associated with the myosin component (*238*), the demonstration of ATPase activity in tissues has frequently been taken as the evidence for presence of myosin or myosin-like systems, particularly in studies on the development of tissues and on the comparative aspects of muscle biochemistry. It is now realized that all tissues possess enzymes capable of hydrolyzing ATP but that the characteristic myosin-type ATPase is associated only with those structures possessing a contractile function.

In the muscle cell, two distinct ATPases have been identified: (*a*) the myosin ATPase which is found only in the myofibril and is associated in some way with the contractile function of this structure; (*b*) the ATPase associated with the sarcosomes and possibly other formed elements within the sarcoplasm. This latter enzyme is not present in the myofibril. This enzyme has been called the soluble ATPase, to distinguish it from the myosin ATPase, but it is preferable to designate it as the granular or sarcoplasmic ATPase (*2a*).

Other distinct ATPases may be present in the muscle cell, for example in the nucleus (such an enzyme has been shown to be present in liver nuclei) (*239*). As yet nothing is known about ATPases in muscle other than the two described above—in any event if others are present their contribution to the total ATPase activity of the cell will be very small.

A. MYOSIN ADENOSINE TRIPHOSPHATASE

A highly characteristic feature of the myosin molecule is its ability to split the terminal phosphate from ATP.

$$\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4$$

Several claims for the separation of the ATPase activity from myosin have been made (*240, 241*) but a clearly convincing demonstration of the existence of the enzyme as a separate protein has yet to be published, and the most satisfactory hypothesis is that the active center or centers concerned with enzymatic activity are integral parts of the myosin molecule. It is true that compared to the enzymatic activity of many purified enzymes that of ATPase is low, but under favorable conditions Q_p* values of 15,000 (equivalent to a turnover number of 4700) have been recorded (*240, 242*). Enzymatic activity remains associated with the H-meromyosin subunit produced on controlled tryptic breakdown of L-myosin.

The enzyme is most aptly described as a nucleoside triphosphatase, for although it is absolutely specific for the triphosphate group the nucleotide base can vary within wide limits as ITP, UTP, GTP, and

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* Q_p, microliters of orthophosphate liberated from ATP by 1 mg. of the enzyme protein per hour.

CTP† (*175, 243, 244*) are all rapidly hydrolyzed. Inorganic polyphosphate is slowly hydrolyzed (*159, 179*).

Usually with enzymes which have ATP as substrate magnesium is the activating ion, but L-myosin is unusual in that it is activated by calcium and magnesium strongly inhibits calcium activation (*119, 245*). Although the ATPase activity of actomyosin at higher ionic strength is very similar to that of myosin with regard to the effects of divalent cations, at low ionic strength ($\mu = 0.05–0.10$) both calcium and magnesium activate the myofibrillar ATPase and the ions are not mutually antagonistic (*246*). Magnesium activation of actomyosin is particularly sensitive to ionic strength and it has been suggested (*119, 247*) that such activation is characteristic of the gel state, while in the sol state the characteristics of cationic activation are identical to those of myosin. When actomyosin has been extracted the magnesium activation of the ATPase is more sensitive to ionic strength than that of actomyosin, as it occurs in myofibrils or glycerated fiber fragments.

Deductions upon the enzymatic capabilities of the myosin ATPase *in situ* which are based on the properties of extracted L-myosin and actomyosin can be misleading (*245, 248*). From the results of enzymatic studies on isolated myofibrils under the ionic conditions presumed to apply *in situ*, it appears that the myofibrillar ATPase can be strongly activated by both calcium and magnesium. In the contraction-relaxation cycle Mg appears to be of particular significance. This cation is essential for optimum contractile effects with ATP both in glycerated fiber and isolated myofibrils systems. Although Ca activates the myofibrillar ATPase, in the absense of Mg contraction does not occur (*248a*). Mg is likewise essential for relaxation (see Section VIII).

From the numerous studies which have been made on ATPase activity of myosin systems [see reviews (*2a, 97, 102, and 116–118a*)] it is apparent that the enzymatic behavior is profoundly affected by the ionic strength, physical state of dispersion of the system, activating cation, relative concentrations of activating cation and substrate, amount of actin present, and often by the previous history of the preparation.

Further evidence as to the apparent complexity of the enzymatic mechanism of ATP hydrolysis by myosin systems is presented by the effect of ethylenediaminetetraacetate (EDTA) which can act, depending on the ionic conditions, both as activator (*244, 249–250a*) and as an inhibitor (*246, 250a, 251*). Fundamental similarities in mechanism of myosin and mitochondrial or granular ATPases exist in that both can be stimulated by 2, 4-dinitrophenol (*252–255*) both are —SH enzymes, and both catalyze the exchange of P^{32} between inorganic phosphate and

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† Inosine triphosphate, uridine triphosphate, guanidine triphosphate, and cytidine triphosphate.

ATP [(*256, 257*), cf. Weber (*258*), Koshland (*259*)]. In the experiments reported by the Ulbrechts (*256, 257*) it is significant that for myosin to catalyze the exchange of inorganic P between ADP^{32} and ATP both actin and Mg must be present.

Under optimum conditions of ionic activation at pH 7.4 isolated rabbit skeletal myofibrils will hydrolyze ATP at a rate not much below that estimated for inorganic phosphate production during maximal activity in mammalian muscle (*2a*). This figure is based on measurements of ATPase activity for 5 minutes or more, i.e., the stationary rate. As there is now good evidence (*259a, 259b*) that the initial rate of actomyosin ATPase activity may be 5 times as great as the stationary rate it may be concluded that the myofibrillar ATPase is potentially capable of accounting for the inorganic phosphate production in muscle during activity.

It is difficult from the data scattered in the literature to compare on a completely satisfactory basis the ATP-hydrolyzing potential of the contractile systems of muscle tissues from various species. The ATPase activities reported for rabbit myosin itself vary quite appreciably from report to report; few accounts of the preparation of L-myosin from other species exist (see Section IV,B), assay conditions are far from constant, and probably the preparations tested were actomyosins of varying actin content. Bailey (*141*) carried out a systematic comparison of enzymatic activity of (acto-) myosin from several vertebrate species and showed that myosin from skeletal muscle of the rabbit, pig, horse, and chicken was more active than its equivalent prepared from the amphibians, frog and toad; also cardiac (acto-) myosin was less active than that prepared from skeletal muscle. The physiological activity of the muscle bears more correlation to the enzymatic activity than the position of the animal from which the muscle is derived among the natural orders of the species. Actomyosin from the housefly, *Musca domestica* (*260*), and from locust thoracic and femoral muscles (*104*) possesses roughly comparable *Qp* values (Ca-activated) to rabbit myosin whereas swine esophagus actomyosin (*144a*) and mammalian cardiac myosins are considerably less active as ATPases (*140, 142*). Needham and Cawkwell (*146*) found mammalian uterine actomyosin from the pig, rabbit, and rat to be 3–10% as active as the same protein isolated from skeletal muscle. Short treatment of uterine actomyosin with trypsin produced a 700% increase in ATPase activity. From the digest a fraction could be isolated (*132b*) which sedimented after 2 hours centrifugation and had ATPase activity comparable to that of H-actomeromysin prepared from rabbit skeletal muscle.

Insect metamorphosis involves considerable changes in the intensity of muscular activity at different stages of development and these are paralleled by similar changes in the enzymatic activity of actomyosin isolated from the larva, the pupa, and the adult insect. For example, in the case of the housefly, *Musca domestica,* Maruyama (*260*) has shown that during the few hours required for emergence of the fly from the puparium the Q_p values of actomyosin isolated from total breis of the insect increased tenfold. Similar changes in the specific activity of total breis (actomyosin plus granular ATPase) have also been observed during the metamorphosis of the wasp *Vespula lewisii* (*261*).

Investigations carried out on the lamellibranchs, *Mytilus edulis, Ostrea edulis, Pinna nobilis* (*262*), have demonstrated the presence of both ATP and actomyosin in the adductor muscles of these mollusks. Usually actomyosin from smooth molluscan adductors has been reported to possess very low ATPase activity (*262–264*). It seems likely in view of the similar solubilities of the two proteins that, unless special precautions are taken during the preparation, such actomyosins will contain large amounts of globulin tropomyosin and hence misleading results will be obtained for ATPase activity and the ATP sensitivity of the sol. Significantly when precautions are taken to remove tropomyosin, actomyosin preparations from both smooth and striated adductors of *Pecten maximus* possess similar enzymatic activities comparable to, although somewhat lower than, that of rabbit myosin (*156*).

ATPase studies on (acto-) myosin of the Bailey (*141*) type isolated from adductor muscles of other lamellibranchs, *Saxostrea commercialis* and *Mya arenaria,* also strongly suggest that the enzyme is of the myosin type in that calcium activates more strongly than magnesium, —SH groups are essential for activity and the pH optimum occurs in the alkaline region (*263, 264*). In the phylum annelida an actomyosin-like protein with the characteristic ATPase activity and contractile properties has been isolated from the echiuroid *Urechis unicinctus* (*265*).

B. Granular or Sarcoplasmic Adenosine Triphosphatase

A number of workers have observed that the aqueous (myosin-free) extract of skeletal muscle possesses ATPase activity (*266, 267*), but Kielley and Meyerhof (*268*) first isolated an ATPase from rabbit muscle which was clearly distinct in physical properties from myosin and was, unlike L-myosin, activated strongly by magnesium. Subsequent studies (*269*) identified the Kielly-Meyerhof ATPase with the small granules (mitochondria and microsomes?) which could be sedimented from homogenates of rabbit and rat muscle from which the myofibril and nuclear fraction had previously been removed by slow-speed centrifugation.

In its characteristics, the enzyme isolated from muscle sarcoplasm resembles the mitochondrial ATPase of liver and other tissues. For example, as with liver mitochondria, granular ATPase activity can be stimulated by 10^{-4} M 2, 4-dinitrophenol if sarcosomes are isolated from rabbit skeletal muscle (*165*) and pigeon breast muscle (*55, 270*) with extreme care. The stimulation is not so great as that obtained with fresh liver mitochondria but it suggests that at least part of the granular ATPase of muscle is of mitochondrial origin. Several workers have noted that both in vertebrate skeletal muscle (*269*) and insect flight muscle (*271*) the ATPase activity of sarcoplasm is not completely removed under conditions which would be expected to sediment mitochondria. It appears that in homogenates of muscle the enzyme is associated with particles of a wide range in size, but it is not clear whether the smaller particles possessing ATPase activity actually exist *in situ* or are the result of the breakdown of mitochondria during homogenization. In this respect, it may be of some significance for comparative investigations of the intracellular distribution of this enzyme that the size and abundance of sarcosomes varies widely in different types of striated muscle. The granular ATPase of vertebrate and insect skeletal muscle resembles myosin in that it is inhibited by —SH reagents (*268, 272, 273*) but there is some evidence of a wider specificity insofar as it can slowly hydrolyze nucleoside diphosphate as well as the triphosphate. For this reason it has been suggested that the enzyme would be more appropriately called a nucleotide pyrophosphatase (*273*).

There are certain difficulties in comparing the relative ATP-splitting activities of the myofibrillar and granular enzymes in muscle, for the activities of these two enzymes respond differently to pH and inorganic ions. The limited studies carried out in which assays of the two enzymes were made under the same conditions would suggest that with increasing activity of striated muscle the relative proportion of the granular ATPase also increases. In rabbit skeletal muscle the myofibrils account for approximately 80% of the total ATPase activity of the tissue (*269*); they account for more than 75% of the total ATPase activity in the femoral muscle of the locust *Locusta migratoria*, but only for 30–50% in the more active flight muscles of this insect (*104*). Active muscles contain more mitochondria than inactive ones, and it seems likely that the higher content of granular ATPase in these muscles is simply a reflection of this fact and that the oxidative metabolism in such muscles is greatly increased.

ATPase activity other than the myosin type has also been reported for both vertebrate and invertebrate smooth muscle. In rat uterus at least 60% of the total activity of the homogenate is associated with the particulate fraction (*146*). A so-called "soluble" ATPase of the oyster, *Saxostrea commercialis*, has been briefly described (*263*) and distinguished from (acto-) myosin ATPase.

The magnesium-activated granular ATPase of locust has been shown to possess a particularly high Q_{10}[*] (*274*) and likewise the apyrase activity of homogenates of thoracic and leg muscles of the cockroach *Periplaneta americana* have also been shown to be markedly temperature-dependent (*275*). In the latter study, no activating ion was added and presumably both the myofibrillar and granular ATPases present in the whole homogenate were activated by endogenous magnesium. It has been suggested (*274*) that this high Q_{10} provides a biochemical explanation for the marked way in which muscular activity in insects depends on temperature. For example, in many insects flight is not possible at thoracic temperatures below 32° (*276, 277*). According to Gilmour and Calaby (*274*) the highly temperature-dependent magnesium-activated apyrase, which they consider to be the most important energy-releasing mechanism in insect flight muscle, is the limiting factor in setting such a high minimal temperature for flight in these insects.

Marked temperature dependence is not unique to muscle apyrase however, for it is also shown by the magnesium-activated ATPases of myofibrils and liver mitochondria (*254*). The corresponding calcium-activated system is not unusually temperature-dependent, suggesting that a high Q_{10} phenomenon is a feature of enzymatic systems involving magnesium and ATP (*254*). The evidence suggests that the granular ATPase is part of the mitochondrial system and unlike the myofibrillar ATPase has no special relation to muscle contraction. Presumably the mitochondrial enzyme is concerned in some way with the oxidative phosphorylating process which provides the ATP for utilization by the myofibril. Therefore if *in vivo* both systems are magnesium-activated, at low temperatures the rate of ATP production in the mitochondria and utilization in the myofibril will be very greatly reduced. Hence the initial warming up of the thorax, achieved by rapid low-amplitude fluttering of the wings, will be an essential step to ensure the rapid ATP production and utilization essential for intensive muscle activity.

VII. Contraction and Adenosine Triphosphate

The concept of the hydrolysis of ATP as the energy-providing step of the mechanochemical process springs from the demonstration (*238*) that most of the ATPase activity of muscle is associated with the (acto-) myosin component. The development of muscle biochemistry is marked by a sequence of abandoned theories of chemical mechanism but, despite critical examination of the facts imposed by this background, with isolated exceptions (*278*) few workers would deny that the interaction of ATP and actomyosin is closely associated with the process of contraction and relaxation in living muscle.

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[*] Q_{10}, the factor by which the velocity is increased on raising the temperature by 10°.

The results of comparative studies indicate that myosin and actin are found in all muscle tissues and in general are remarkably similar in properties whatever the source. Significantly there are suggestions that actomyosin-like protein which possesses contractile properties occurs in tissue and organisms other than muscle (*279, 280*). Although actomyosin is unique to contractile tissues, ATPases and nucleoside triphosphatases (NTPases) are more widely distributed. Other NTPases have not been studied as intensively as the myosin enzyme and further information is necessary in some cases as to whether their specificity is strictly comparable, yet it appears that the ability to liberate the terminal phosphate from nucleoside triphosphates is not a unique property of the contractile system. It is possible that the mitochondrial ATPase may have some function associated with contractile behavior in mitochondria (*270, 272*) but this enzyme is probably implicated in other systems and partly purified preparations bear no resemblance to myosin. Many features of hydrolysis of ATP by actomyosin are also shared by the mitochondrial ATPase (*254, 255*), and studies with P^{32} indicate that the underlying mechanism of ATP hydrolysis in both systems is similar (*256, 257*). The interaction of actomyosin with ATP is unique, however, in that in the gel state it brings about contraction of the protein system. With such ATP-induced contraction, hydrolysis invariably occurs. It should be pointed out that contraction can also be induced by the other nucleoside triphosphates, UTP, GTP, ITP, and CTP, small amounts of which have been shown to be present in muscle. It is possible that *in situ* any of the nucleoside triphosphates could be concerned in the contractile process as they will be maintained in equilibrium with each other by the nucleoside diphosphokinase enzyme. The relatively much higher concentrations of ATP in living muscle and its unique position as both a contracting and relaxing agent in muscle models (*175, 258*) strongly suggest that it is the triphosphate concerned.

Although, as has already been stated, most workers would agree that ATP is directly involved in the contractile process, it is a matter of controversy as to the precise point at which ATP is dephosphorylated during this process. One view championed particularly by Weber (*258*) is that contraction cannot take place until ATP has been dephosphorylated, as it is considered that the contractile element must first be itself phosphorylated at the expense of ATP before it can shorten. The other view which has been ably defended by Morales and collaborators (*116, 281*) is that it is only necessary for ATP to be bound by the contractile system; this process is considered sufficient to neutralize the extending charges on the element so that shortening will then occur due to the forces of thermal agitation (see Section IX). In the latter hypothesis, the transfer of chemical into mechanical energy occurs at the binding process and ATP hydrolysis is not essential but merely part of the recycling process (*281*).

A. *In Vitro* STUDIES

By virtue of the fact that in model systems it is easier to study and control the turnover of ATP than with the intact tissue it might be considered that *in vitro* studies offer more promise for elucidating the mechanochemical process. The evidence in support of the splitting-contraction hypothesis has been presented by several workers (*2a, 97, 163, 282*) and is mainly of the kind demonstrating a parallelism between the rate of tension development and the hydrolysis of ATP under a variety of conditions in model fiber systems. One type of evidence of a rather different kind is that an extremely low concentration of ATP, which alone is not able to bring about contraction, is extremely effective in doing so if it is supplemented by a phosphokinase system such as creatine phosphokinase (*229*) or pyruvic phosphokinase (*283*). Systems of this type enable a fixed concentration of ATP to be maintained and suggest strongly that its rate of turnover rather than initial concentration is the important factor for contraction.

A few workers (*284, 285*) have reported examples of a lack of parallelism between rate of contraction and ATPase activity in glycerated fiber models. For example under certain circumstances the addition of 0.3 *M* KCl was shown to reduce the rate of ATPase activity by 80% and increase the contraction rate by 20%. Also it has been claimed that inorganic triphosphate, although split by actomyosin at an appreciable rate, is relatively ineffective at inducing contraction (*179*). Evidence of this kind, together with theoretical arguments which are more difficult to refute, is presented in several reviews by Morales and collaborators (*116, 281*).

It is worth pointing out that arguments based on the nonparallelism of contraction and splitting under certain conditions need not necessarily be serious objections to the hypothesis that contraction involves ATP hydrolysis. A more serious objection, for which there is as yet no evidence, would be that an ATP-induced contraction can take place in the absence of hydrolysis. It is evident that mechanical response of actomyosin is much more sensitive to changes in ionic strength than is

ATPase activity. In the extreme case where actomyosin is in the sol state, ATP is hydrolyzed but contraction does not occur and only a dissociation of the protein complex takes place. When it is considered how sensitive to ionic strength is the physical state of actomyosin gels, and how difficult it is to decide what are the precise conditions existing at any point within a glycerated fiber because of permeability consideraations, it is perhaps surprising that such good agreement between contraction and splitting is generally obtained in model systems.

Although as yet there has been no demonstration of ATP-induced *in vitro* contraction occurring without splitting, so-called contraction of model fibers without ATP by ions such as SO_4^{2-}, $Fe(CN)_6^{2-}$ (*286*), I^-, and SCN^- (*287*), and high Ca^{2+} concentrations (*288*) have been reported. It is likely that such effects are nonspecific and bear no relation to physiological contraction (*282*).

In the opinion of the author the experimental findings suggest that whenever contraction occurs ATP is hydrolyzed but they do not finally allow us to decide whether the transfer of energy to the contractile system occurs during ATP binding or at the moment of dephosphorylation. It may be that these two events are so closely associated that normal experimental techniques will not separate or distinguish them. As seen by Weber and collaborators on one hand and Morales and his associates on the other, these two hypothesis involve basically different mechanisms in that one requires covalent bond formation and change in chemical potential, whereas the other utilizes entropic forces to bring about contraction (see Section IX). Recent demonstration of the equilibration of P^{32} between ADP and ATP by actomyosin systems (*256*, *257*) can be interpreted as evidence for the formation of a phosphorylated protein intermediate such as is demanded by the hypothesis postulated by Weber (*258*).

B. *In Vivo* Studies

From *in vitro* studies there is no doubt that ATP can induce the contraction of actomyosin systems. This can be demonstrated with well-washed isolated myofibrils where ATP is the only nucleotide present apart from the adenine nucleotides bound to the myofibrillar structure (see Section V,B). In this system ATP is hydrolyzed and in consequence its level falls as contraction occurs. The aim of *in vivo* studies has been to show that in the early phases of the single twitch a fall in ATP level compared to that of the control can be demonstrated. Implicit in experiments of this kind is the assumption that the rate of utilization of ATP is very much greater than the *initial* rate of reformation in the tissue during a single twitch. Such an assumption may not be completely justified, for the evidence indicates that the mechanisms for ATP breakdown and reformation are both enzymatic and may be expected to possess comparable velocities. It may well be that at the beginning of a period of activity in muscle, i.e., during a single twitch, rephosphorylation reactions are extremely fast.

The technical difficulties in this type of investigation are considerable. The muscle must be fixed instantaneously at a precise point in the contraction cycle, the control must be likewise fixed without stimulation, and the analysis involves the separation and accurate estimations of small differences of the various phosphate fractions in the tissue. A number of workers have attempted to answer this question with living muscle but the results have been somewhat variable. A fall in the total polyphosphate fraction and an increase in the ADP level has been reported after a single twitch or a very short period of contraction in leg (*289, 290*) and rectus abdominus (*291*) muscles of the frog, and rectus abdominus muscle of the tortoise (*292*). More detailed investigations employing frog rectus abdominus muscle (*293*) and turtle leg muscles (*234*) indicated fairly decisively that no change could be detected in the concentration of the adenine nucleotides and creatine phosphate after or during a single twitch developed at temperature close to 0° under aerobic and anaerobic conditions. It may be significant that at 20°, when muscle performs appreciably more work, a decrease in the ATP level can usually be demonstrated. Although regeneration of ATP by aerobic processes or by the creatine phosphokinase system was excluded in these experiments, it is possible that some anaerobic regeneration of ATP occurred as a result of glycolysis stimulated by the liberation of inorganic phosphate generated at the instant of contraction.

Inorganic phosphate was produced during a single twitch at the expense of some unknown precursor which was in the fraction representing the total organic phosphorus less that due to creatine phosphate (*293*). No further information is as yet available on this precursor. It is questionable if the amounts of phosphate produced, which were not much greater than the standard error of the estimation, are adequate to account for the work done, assuming 100% efficient conversion and the newly determined ΔF° for ATP hydrolysis (*294*). This fact could be taken to indicate that some of the phosphate liberated by the twitch had already been reconverted to adenine nucleotides.

The observation that the state of oxidation or reduction of pyridine nucleotide of the mitochondria is controlled by the level of phosphate acceptors such as ADP, has been used by Chance and Connelly (*295*) to follow by spectrophotometric means the ADP changes which occur during contraction in the perfused frog sartorius muscle. They conclude that although a progressive increase in the ADP concentration accompanies repeated stimulation, the amount of ADP produced per twitch is only 2–3% of that which would be expected if ATP were the sole source of energy. Such spectrophotometric studies can only measure changes in the level of ADP which is available at the sarcosomal membrane and which can exert its influence through it. One must take into account the fact that any ADP formed will also be rephosphorylated in the sarcoplasm where it is produced, by glycolytic and creatine phosphokinase systems. The results of Chance and Connelly are of importance in that they confirm that an increase in ADP does occur after a single twitch, but they cannot be used to determine the amount of ADP produced by such a contraction because they do not take into consideration the contribution of anaerobic phosphorylating systems.

The application of tracer techniques would seem to offer a promising method for the study of the turnover of the organic phosphates of muscle during activity but certain difficulties arise in the interpretation of the results. Several workers (*295a,b*) have been unable to demonstrate the equilibration of phosphate between the adenosine polyphosphates and creatine phosphate which might be expected if rapid breakdown and resynthesis of ATP occurs during activity.

VIII. Relaxation

If well-washed isolated myofibrils or glycerated fiber preparations are suspended in a medium containing inorganic salts and ATP of concentrations comparable to those presumed to exist within the cell, marked contraction occurs. This effect, which is accompanied by a high rate of ATP hydrolysis, is obtained with Ca and Mg present together over a wide range of concentrations, and with Mg alone over a more restricted range, but not with Ca as the only activator. Analysis suggests that in the resting muscle cell, the rate of inorganic phosphorus production is low, and one is forced to conclude that *in situ* there exists some factor or factors which prevent ATP from inducing contraction in resting muscle but whose effect instantly disappears on stimulation. It seems likely that the factor acts by inhibiting the ATPase activity of the myofibril, for invariably in model systems relaxation is associated with a relatively low rate of ATP hydrolysis.

From earlier studies by many workers on actomyosin the view has emerged that ATP has two distinct effects. In the first place, it is considered that ATP breaks down the interaction between the actin and myosin moieties of the complex—the so-called plasticizing effect of ATP. In the sol state this is the only apparent change and results in the characteristic fall in viscosity, whereas in the gel state filaments previously locked to other filaments by the interaction can now move relative to one another with a consequent fall in the tensile strength of the system. Secondly in addition to its plasticizing action, ATP can also bring about contraction of the gel system. This effect is associated in some way with a relatively high rate of hydrolysis of ATP or other nucleoside triphosphates, whereas plasticizing action can occur in the absence of nucleotide breakdown.

Although well-washed glycerated fiber preparations do not spontaneously relax after contraction by ATP under isotonic conditions, nevertheless relaxation has been demonstrated in these fibers by subsequently treating the system with an ATPase inhibitor such as Salyrgan (Hg-salicyl-allyl-amid-sodium acetate) under carefully controlled conditions (*97, 163, 168*) or with EDTA (*296–298*). In such systems the ATPase is strongly inhibited and the plasticizing effect of ATP becomes dominant; the tension falls with the result that relaxation occurs.

All groups on the ATP molecule are apparently not essential for plasticizing action because relaxation can be obtained in glycerated fibers by washing out the ATP after contraction and replacing it with low concentrations of pyrophosphate and tripolyphosphate (*97, 163, 296, 299*). This effect is presumably related to the action of these substances in dissociating actomyosin (*159, 176*). Under certain conditions, pyrophosphate can induce relaxation in the presence of ATP (*300*), and in some cases the function of the pyrophosphate seems to be to inhibit the ATPase activity by binding the Mg-activating ion (*246*) so that the relaxing action of the ATP itself becomes apparent.

The above studies are not strictly comparable with relaxation under physiological conditions but, so far as they indicate enzymatic mechanisms which may be involved in the process, they are of value as model systems. Likewise of importance in understanding the mechanism of relaxation is a knowledge of the factors affecting the ATPase activity of the myofibril. Enzymatic studies on isolated myofibrils reveal the following characteristics of the ATPase system which are of importance in analyzing model systems of relaxation:

(*a*) High concentrations of ATP inhibit the Mg activated ATPase (*246, 301*).

(*b*) Substrate inhibition by ATP is dependent on the concentration of Mg (*246, 282*).

(*c*) Substrate inhibition is not obtained when Ca is the activating ion and low concentrations of Ca can relieve the substrate inhibition obtained when Mg is the activator (*246*).

Considerable difficulties attend analysis and description of the ATPase system of the glycerated fiber models used in relaxation studies, for unless the fiber is extremely thin it is difficult to ensure that the substrate concentration and ionic conditions are exactly the same outside the fiber and at all points within it. Also the precise relation between substrate inhibition and the relative concentrations of Mg and ATP is somewhat dependent on the previous history of the myofibrils or glycerated fibers (*246*).

Evidence for relaxation induced by ATP itself without the addition of artificial plasticizers or nonphysiological means of lowering the ATPase activity was first obtained by Bozler (*302*) with glycerated fibers. He showed that application of ATP at higher concentrations to briefly washed fibers which had been stored in glycerol for a short time produced contraction followed spontaneously by relaxation. With old well-washed fibers ATP produced contraction only. Simultaneously Marsh (*303, 304*) demonstrated the existence of a factor in muscle homogenates which inhibited ATPase activity; in the presence of this factor, the addition of ATP to homogenates of rabbit muscle caused a lengthening of the cell fragments of which the homogenate was composed. In the absence of the factor, ATP caused the pronounced shortening of the cell fragments which is characteristic of the normal action of this nucleotide on washed actomyosin systems. Marsh's factor was rather labile but in Bendall's (*305, 306*) hands the preparation was apparently more stable, and he showed that in the presence of ATP and Mg it would induce relaxation in well-washed glycerated fiber preparations (Fig. 11). In general, studies on relaxation in model systems (*306, 307*)

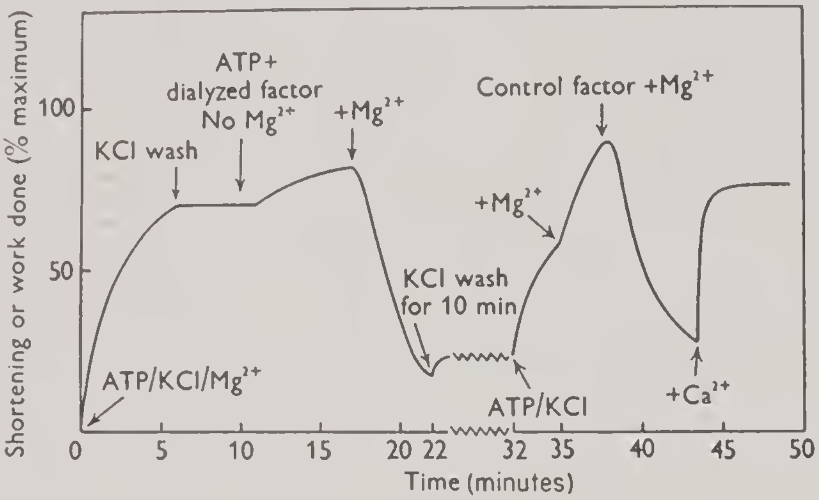

FIG. 11. Effect of dialyzed relaxing factor on the ATP induced shortening of glycerated fibers in the absence and presence of magnesium and calcium. (After Bendall, *306*.) The control factor was an undialyzed sample from the same stock as the dialyzed. Concentrations: 6 mM ATP; 0.16 M KCl; 4 mM MgCl$_2$; 0.2 mM CaCl$_2$.

have shown that a low rate of ATP hydrolysis by glycerated fiber fragments is characteristic of relaxation.

A number of protein fractions isolated from rabbit skeletal muscle have been claimed to possess relaxing action. These include creatine phosphokinase (*308–310*) and myokinase (*311*) although Japanese workers consider that, in order to be effective, both phosphokinase systems must be supplemented with sarcosomes or some extract from these granules (*312, 313*). The granular preparation which has been suggested as implicated in the system (*313*) is rich in ATPase activity.* Presumably other workers have earlier failed to detect a requirement for the granular fraction because unless special precautions are taken, the usual glycerated fibers will contain sarcosomal material. Using isolated myofibrils as a test system Portzehl has shown that relaxing activity of rabbit sarcoplasm can be quantitatively recovered from the granular fraction sedimented at 35,000 *g* (*313b*).

Analysis of relaxing systems and identity of the factor or factors presents many difficulties but nevertheless certain facts appear to be of significance if we consider that relaxation occurs when the myofibrillar ATPase activity is reduced to a low level. These are as follows:

(*a*) In the presence of relaxing factor preparations the concentration at which ATP becomes inhibitory for a given set of ionic conditions is lowered (*301*).

(*b*) The relaxing effects of phosphokinase systems such as myokinase and creatine phosphokinase are related to their ability to maintain the ATP level within the fiber at the inhibitory level at the expense of substrates which can readily penetrate into the fiber (*2a, 314*). If relaxing factor is present in addition to the phosphokinase, the ATP level required to produce relaxation may be quite low (*310*).

(*c*) The essential nature of Mg for relaxation (*306*) and the plasticizing action of polyphosphates in general (*296, 299*).

(*d*) The marked inhibition of relaxation by low Ca concentrations (*306, 315*).

In summary, it may be said that in the muscle there exists a factor or factors which maintain the myofibrillar ATPase systems in a state of inhibition although the over-all concentrations of Ca, Mg, and ATP in muscle are such as would induce, if each was effective, a rapid rate of ATP hydrolysis. Phosphokinase systems by maintaining the ATP level may potentiate the effect but there are suggestions that the relaxing factor system is not identical with either creatine phosphokinase or myokinase (*312, 313b, 314*).† Whatever the nature of the factor, stimulation of the muscle instantly causes it to lose its effect and permit the contracting action of ATP to take place. Interpreted at the enzymatic level, a momentary release of free Ca at low concentration would effectively relieve the substrate inhibition and cause a sharp increase in the level of ATPase activity (*2a, 163*). As yet, however, the mechanism linking the electrical changes at the membrane and the enzymatic events at the myofibril remains completely in the realm of speculation. For further discussion on relaxation see review articles (*2a, 118, 163*).

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* The ATPase and relaxing factor activities of the granules are both abolished by treatment with phospholipase; however, certain treatments destroy the relaxing factor activity but not the ATPase activity (*313a*).

Practically all investigations on relaxation have been carried out using glycerated fibers from rabbit psoas and factor preparations from skeletal muscle of the whole animal. The evidence for basic similarities in the construction and mechanism of the contractile systems is strong and it might reasonably be expected that likewise the mechanism of relaxation is similar, at least in striated muscles. It is conceivable that relaxation in smooth muscle with the characteristic slow decay of tension is controlled in a somewhat different way. Hardly any comparative work of this kind has yet been done; however Bendall (*311*) demonstrated relaxing-factor activity towards rabbit glycerated fibers in horse psoas extracts. Somewhat surprisingly, he was unable to detect relaxing-factor activity in similar extracts from horse and rabbit heart, rat leg muscles, and rat uterus. Bendall's results may indicate the difficulty of detecting factor activity with the system used, rather than its absence from these tissues. A granular fraction can be isolated from pigeon breast muscle which inhibits the myofibrillar ATPase and which is presumably similar to the relaxing factor preparation from rabbit skeletal muscle (*315e*).

IX. Mechanisms of Contraction

Mechanical change in the muscle cell is brought about by a decrease in length of fibrillar units orientated roughly along the axis of contraction. At the myofibrillar level changes in striations are not characteristic of the contracted state in smooth muscle, for cross striations comparable to the A and I bands of striated muscle are not a feature of this tissue. In smooth muscle, therefore, changes in the myofilaments which bring about shortening must occur in such a way that the pattern as seen with the light microscope remains unchanged. In striated muscle however, changes in the cross striations are characteristic features which can be correlated with definite stages in the contractile cycle. Although there has been some controversy as to the precise nature of the band changes, the present evidence strongly suggests that they correspond to those illustrated in Fig. 12 (*17, 39, 316*).

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† Although all workers now agree that granular or possibly reticular material isolated from the sarcoplasm is an essential component of the relaxing factor system there are differences as to what additional factors are required to give maximal activity. Some workers (*313a, 315a*) maintain that a transphosphorylase system is required to potentiate the relaxing activity of low concentrations of the granules. Others (*315b,c,d*) consider that transphosphorylase systems have no specific role other than maintaining the ATP level but have presented evidence for the presence of a dialyzable cofactor in relaxing factor systems. Pyrophosphate has some properties similar to those of the cofactor but is not identical with it (*315d*). It is clear that further work is needed both to define the relaxing factor system and to elucidate its mode of action.

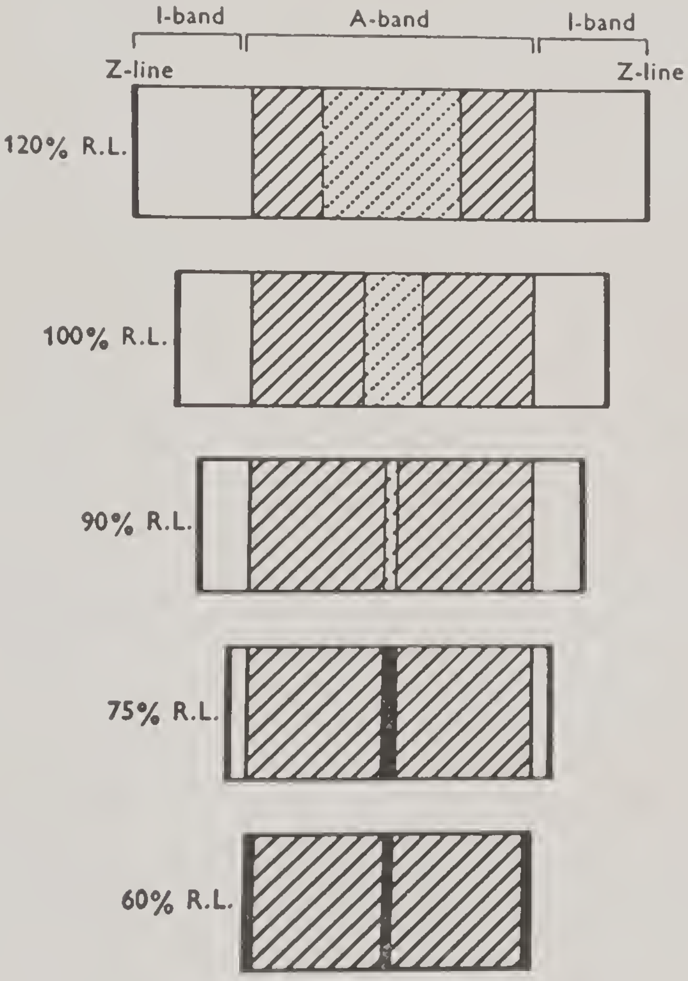

FIG. 12. Band changes in the striated myofibril during contraction. [After Hanson and H. E. Huxley (*17, 324a*).]

At resting length the rabbit skeletal sarcomere is about 2.3–2.5μ long, the I band slightly shorter than the A band in which the H space is apparent. Contraction down to 60–70% of the rest length involves a gradual shortening of the I band which in the first stages of the contraction is accompanied by the disappearance of the H space. As the I band finally disappears, the Z band comes into close contact with the outside edges of the A band. Throughout this range of contraction the length of the A band has remained unchanged, but on further shortening the edges of the A band fuse with the Z and the over-all appearance is of regular dense bands in the Z band area—the so-called reversal of striation. The sarcomere length is now shorter than the original resting length of the A band and apparently the A substance is also concentrated at the ends of the shortened sarcomere.

Theories of contraction have recently been reviewed and evaluated in relation to the known properties of mucle by Wilkie (*317*). Obviously any theory of contraction must ultimately be assessed on the basis of whether it provides a satisfactory explanation of the structure and physiological characteristics of the tissue. Here discussion will be confined to the mechanisms postulated for the shortening of the fundamental contractile elements of the myofibril and their relation to metabolic processes.

A. FOLDING MECHANISMS

The simplest mechanism for the shortening of a myofibril built up of longitudinally orientated myofilaments is that the filaments themselves shorten by some folding process. Astbury (*318*) proposed a theory based on changes in the configuration of the polypeptide chain of myosin but both wide and narrow angle X-ray analysis have given little evidence for such a type of folding.

Two main types of mechanism have been proposed to explain the shortening of a protein filament by ATP. One postulates that the contractile element is kept extended by electrostatic charges distributed along its length. Contraction occurs when these charges are neutralized and thermal agitation forces cause a rise in configurational entropy. This theory has been stated in varying forms by several workers (*319–322*) and recently in detail by Morales and collaborators (*116*, *281*) who postulate that negatively charged ATP ions neutralize the extending positive charges on the contractile element. Contraction accompanies this binding and the ATP is subsequently dephosphorylated and comes off leaving the element to re-extend (see Fig. 13).

The other view, championed particularly by Weber (*258*) and also postulated by Binkley (*323*), requires that the contractile element is phosphorylated and thence a covalent bond is formed between different points to bring about a shortening, as illustrated in Fig. 14. This scheme as proposed by Weber (*258*) demands that dephosphorylation of ATP is absolutely essential for contraction, whereas in contrast the Morales standpoint is that binding of the nucleotide itself is enough to bring about this change.

Whatever the process involved it is extremely fast, and as Morales (*281*) has pointed out it is questionable from the chemical kinetic standpoint whether the speed of formation and dissolution of the two covalent bonds required in Weber's scheme is great enough to satisfy this requirement. From this point of view ionic neutralization is a more satisfactory mechanism. If in fact a mechanism of filament folding has to be invoked to explain muscle contraction, some compromise between these two theories may have to be struck which does not demand the separation of dephosphorylation and contraction in such a clear-cut fashion. An example of such a hypothesis has been proposed by Riseman and Kirkwood (*321*), who suggest that phosphorylation of the hydroxy amino acids could change the charge distribution on the contractile element sufficiently to induce a configuration entropy change.

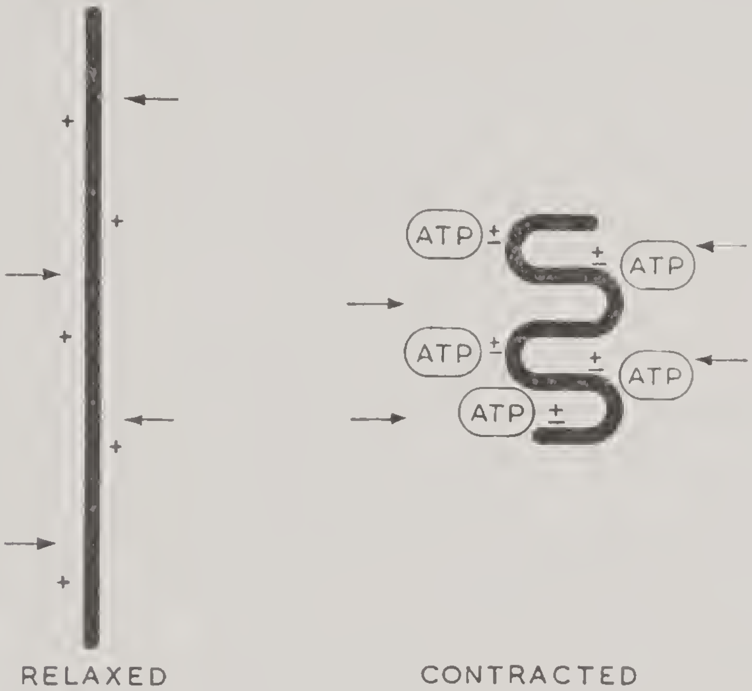

Fig. 13. Scheme of mechanism in which the binding of ATP induces contraction. The contractile element is shown extended in the relaxed condition by the mutual repulsion of positive charges distributed along its length. On binding negatively charged ATP ions these charges are neutralized and shortening takes place as the result of thermal agitation forces. [After Morales (*281*).]

B. Sliding Mechanisms

On the basis of observations made with the interference microscope on the band changes in the living single fiber A. F. Huxley and Niedergerke (*39*) suggested a mechanism of contraction involving two types of filament sliding past each other. Independently, H. E. Huxley and Hanson (*324*) proposed a similar mechanism as a result of investigations with isolated myofibrils contracted with ATP and electron microscope studies of myofibrillar cross sections. According to this hypothesis on contraction the I filaments, which run continuously into the A band between the thicker A filaments, slide further into the A band and bring about the disappearance of the H space (Fig. 15). Further contraction with the disappearance of the I band results as the I filaments are drawn up into the A band, presumably folding as they do so, until the Z lines approach the outside edges of the A band at ~60% contraction (Fig. 12).

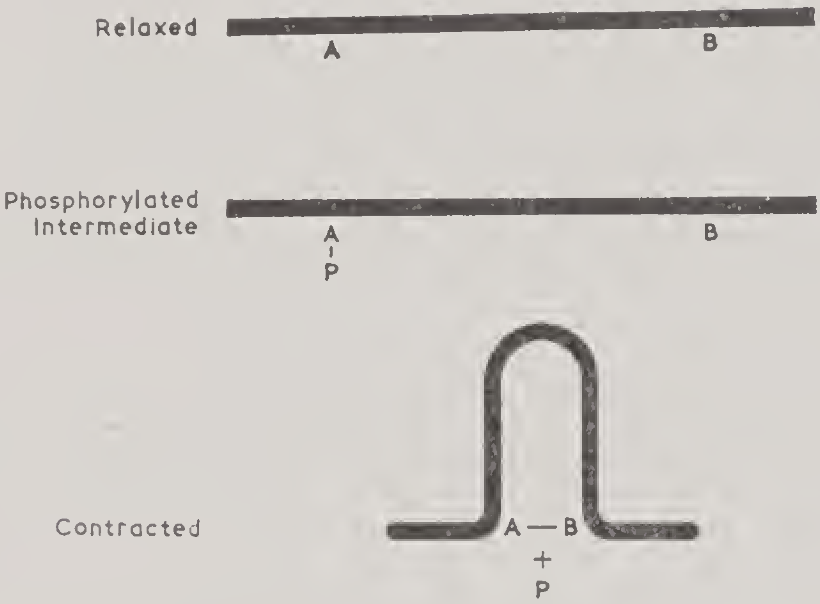

FIG. 14. Scheme of mechanism for contraction involving phosphorylation of the contractile element. A and B are groups which lie some distance apart on the contractile element. A is phosphorylated and thus activated it can form a bond with B. As a consequence a fold is formed resulting in contraction and inorganic phosphate is liberated. [After Weber (258).]

It is a postulate of this hypothesis as expounded by H. E. Huxley and Hanson and A. F. Huxley that the A filaments which do not change in length (at least up to ~60% contraction) consist of myosin, whereas the I filaments which actually bring about the shortening are composed of actin. In view of the amount of tropomyosin present, this protein at least must also be considered as a component of one of the filaments and the evidence suggests that it may be associated with the actin (*106*).

A satisfactory explanation of the mechanism of the movement of the I filaments into A band in terms of what is known of the interaction of actin, myosin, and ATP presents many difficulties. Those put forward so far are of the following types.

(*a*) Actin filaments are drawn in between the myosin filaments to increase the number of points at which the actin-myosin interaction can take place. It is presumed that this occurs when ATP, which has previously displaced actin from myosin, is enzymatically split (*324*).

(*b*) Actin filaments become negatively charged as a consequence of binding phosphate produced by ATP hydrolysis. The myosin filaments are positively charged and consequently the actin filaments are pulled further into the spaces between the myosin filaments by electrostatic attraction (*325*).

(*c*) One filament (actin) cyclically undergoes small reversible changes in length (~2%). At certain stages in the cycle the actin filament is attached to the fixed myosin filament so that the net result is that the actin filament moves along the myosin ("caterpillar-like action"). Such a scheme could be related to actin polymerization and depolymerization (*17, 326*).

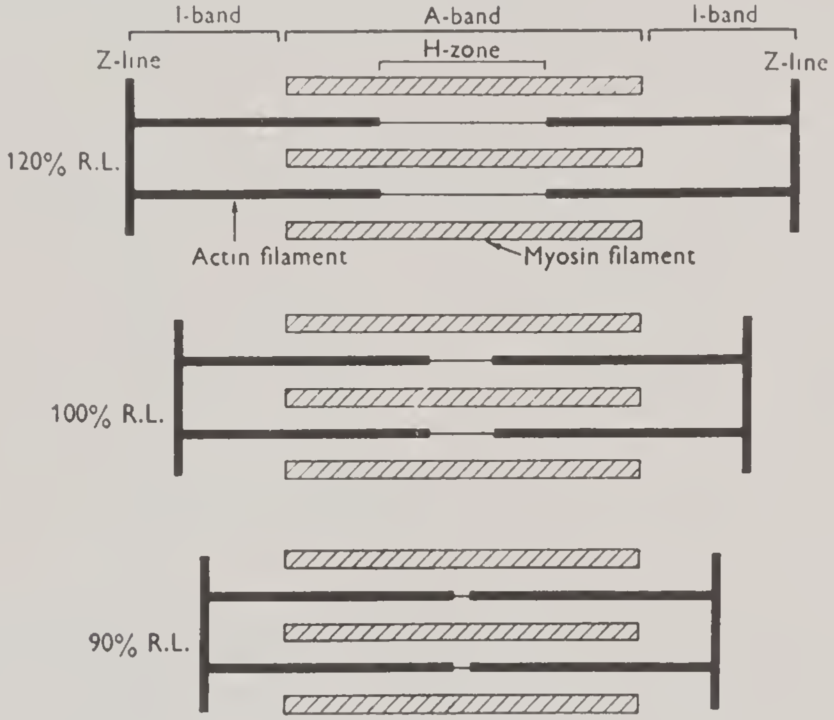

FIG. 15. Scheme showing the behavior of the filaments in the I and A bands during changes in length of the myofibril according to the model proposed by H. E. Huxley and Hanson and A. F. Huxley and Niedergerke. [From H. E. Huxley (*324a*).]

(*d*) The myosin filaments possess contractile branches (*17*) or mating elements (*316*) which at the moment of contraction attach themselves to definite points in the actin filament and by contraction or movement relative to the myosin filament draw along the actin filament relative to the myosin. Each combination draws the actin a short distance, is broken and remade at a point further along the actin filament, and the process is repeated. By an asynchronous action steady tension could be maintained in the actin filaments (*316*). It is of interest that cross links or bridges between the A and I filaments can be observed in electron micrographs of thin sections of striated (*40*) and smooth muscle (*42a*). H. E. Huxley estimates that the number of bridges in the myofibril of rabbit skeletal muscle is of the same order as the number of myosin molecules present.

A. F. Huxley has analyzed in detail a mechanism of the type described in scheme (*d*) in which it is assumed that the combination of actin and myosin takes place spontaneously with the equilibrium in favor of complex formation. The union is broken when a high-energy phosphate compound (XP) unites with a site near A (Fig. 16). Finally XP is split and the products, which include inorganic phosphate, are detached leaving A free to combine with another M group further along the myosin filament; the cycle is then repeated. Analysis of such a mechanism indicates that it accounts fairly well for the mechanical and thermal behavior of muscle (*316*).

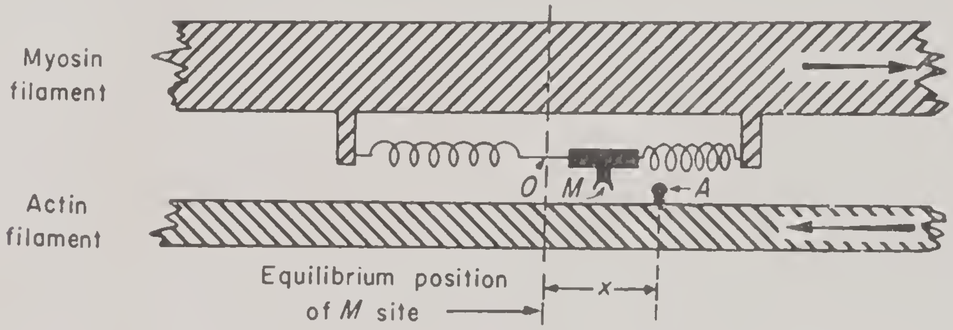

FIG. 16. Diagram illustrating scheme proposed by A. F. Huxley (*316*) to explain the generation of tension and the mechanism of shortening in the striated myofibril. The actin filament is attached to a Z line which would be off the diagram to the right. The arrows indicate the relative motion between the filaments when the muscle shortens.

The sliding mechanism adequately explains shortening down to the point at which the I band disappears, i.e., ~60% which is about the extreme of the physiological range, but the sarcomere can contract beyond this until it is shorter than the original A band. In fact *in vitro* myofibrils can shorten down to 25–20% of their original length. Under these conditions the A band filaments (myosin) must also have shortened in some way and the denseness of the contraction bands apparent in the contracted striated muscle suggests that material is no longer evenly distributed along this band.

Weber (*326a*) has suggested a mechanism to bring about the contraction of a system of interdigitating filaments, which incorporates some of his earlier ideas on the chemical processes involved during the shortening of a single contactile filament. From the results of the investigations carried out by the Ulbrechts (*256, 257*) on the exchange of P^{32} between ADP and ATP catalyzed by actomyosin systems, Weber has concluded that an acid group activated by phosphorylation is localized on the actin. This group forms in succession covalent links with —SH groups and phenolic and alcoholic —OH groups on a parallel myosin filament. In such a scheme, contracting muscle functions thermodynamically like an electric motor and not like a stretched spring.

Sliding mechanisms have the advantage that they would not be expected to produce marked changes in the X-ray diffraction pattern of muscle on contraction over the physiological range. Although the sliding mechanism explains rather well the band changes occurring in striated muscle during contraction, the scheme must also be adaptable to smooth muscle for it is probable that the basic mechanism of contraction is common to both types of muscle. There are, however, difficulties in applying to smooth muscle the sliding mechanism in the simple form proposed for striated muscle. The limited evidence available suggests that a two-filament system is not present in all types of smooth muscle (*42a*) and, where it has been demonstrated, the two-filament systems appear continuous (*42a*, *48*). Contraction in this type of muscle must surely involve folding of the filaments, possibly as a consequence of the interaction between the two filament systems.

C. Other Mechanisms

The sliding mechanism requires the presence of two interlocking sets of different, parallel filaments in the A band. Although the electron microscope evidence of H. E. Huxley is impressive and his investigations have to some extent explained the findings of others, not all workers are agreed on the nature of the fine structure of the A band. Hodge (*29*) in his study on dipteran flight muscle came to the conclusion that myofilaments were continuous throughout I and A bands and other workers (*22*) have provided evidence which implies a similar structure.

Spiro (*41*) concludes from his investigations of thin sections of the striated myofibril that in uncontracted muscle the H band contains rather thick filaments, whereas in the remainder of the A band there are about twice the number of thin filaments which are continuous through the I band up to the Z membrane. In myofibrils contracted down to ~60%, the A band contains only thick filaments. To explain these results Spiro suggests that the thin filaments in the A band consist of actin whereas the myosin is present as fine interfilamentous material which accounts for the density of the A band. When contraction occurs it is suggested that accompanying the splitting of ATP, actin and myosin complex by the deposition of thin molecular units of myosin on the actin filaments. It is presumed that these proteins complex together to form a compound super coil or rod by a process which draws the actin into closer proximity with the myosin and hence pulls the I filaments into the A band. The A filaments now appear thick and Spiro considers that such a coiled complex may account for the hollow appearance of the A filaments observed by several workers (*15, 22, 29, 40*).

X. Conclusions

As yet there has been no confirmed report of the absence from recognized muscle tissue of actomyosin as characterized by the unique coupled mechanical and enzymatic changes evoked by ATP. These facts strongly suggest that this protein complex plays a central role in contraction. It also follows from the limited physicochemical data available that myosin and actin isolated from various species and muscles form homogeneous groups of protein. Although direct evidence is mainly available for the myofibril from skeletal muscle of the rabbit, it seems very likely that in striated muscle at least the contractile apparatus consists mainly of these two proteins associated in some way with tropomyosin.

The interaction of actin and myosin possesses certain aspects which suggest an important function in contraction, but less information is available regarding the role of tropomyosin. Nevertheless proteins of this type isolated from a wide range of species form a remarkably homogeneous group and appear to be much more abundant in muscles of the smooth type in which the actomyosin content is apparently lower than that of the striated counterpart. It is not unreasonable to draw the conclusion that the relative amounts of actomyosin on the one hand and tropomyosin on the other are determined by the particular function of the muscle.

The evidence for the hydrolysis of ATP as an important step in the mechanochemical process is convincing even though it is not acceptable to all workers in the field. Comparative study itself supports this hypothesis in so far as there is some correlation between the enzymatic activity of the actomyosin system and the intensity of activity of the muscle from which it is derived.

The universal occurrence of actomyosin in muscle tissue also adds support to the view that the basic mechanism of contraction is common to both striated and smooth muscle cells. Contraction involves changes in the fine structure of the contractile unit and a general mechanism must imply certain basic features at this level of organization in the contractile systems of both striated and smooth muscle cells. It follows that the striation of skeletal and cardiac muscle which is consequent on the localization of myosin in a periodic fashion along the myofibril is not a basic requirement for the contractile mechanism but rather a development for the fast contraction-relaxation cycle associated with those types of muscle. The interlocking system of two types of filaments is a striking feature of the rabbit myofibril and the mechanism proposed for contraction in such a system demands that actin and myosin should be localized in different filaments. Although there are suggestions from the combined results of electron microscopy and biochemical investigations that there is such a distribution of these proteins, it should be pointed out that direct evidence for it has yet to be provided. A basic mechanism for shortening involving a dual system of sliding filaments demands that two types of discontinuous filaments should be present in smooth as well as in striated muscle. As yet the evidence on this point is inconclusive for although two-filament systems, apparently continuous, have been observed in some types of invertebrate smooth muscle they have not been detected in certain vertebrate smooth muscles.

It must be admitted that we are far from being able to explain the biochemistry of contraction but our knowledge of this process is now approaching a critical stage. The possibility is in sight of being able to describe the fine structure of a functional unit within the cell in terms of the molecules of the substances of which it is composed. This is a vital stage for the understanding of the mechanism of contraction and would represent an outstanding advance in the integration of biochemical and cytological knowledge.

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Addendum

Myosin. Application of the Archibald technique for molecular weight determination has given values in the region of 420,000 for rabbit skeletal myosin (*327, 328*). Similar results have also been obtained with the sedimentation-diffusion method at low protein concentration (*327*). The former technique gives values of 324,000 and 126,000 for the molecular weights for L-and H-meromyosins respectively (*329*). This means that the molecule of myosin is composed of a molecule each of the meromyosins rather than one H- to two L-meromyosins as was formerly supposed. Further evidence which calls to question the homogeneity of the conventional L-myosin preparations has been provided by the separation from myosin dialyzed against 0.1 M Na_2CO_3, of a component of molecular weight 29,000 which represents 14–18% of the original L-myosin (*330*). Also chromatography on diethylaminoethyl cellulose yields fractions in which the specific ATPase activity is not constant (*331, 332*).

Although SH groups are required for both the ATPase and actomyosin forming activities of myosin, Bárány considers that the groups involved may not be identical. This is suggested by the observation that myosin which is without ATPase activity but is still capable of combining with actin can be isolated from iodoacetamide treated actomyosin (*333*).

CHAPTER 6

Other Mechanisms Producing Movements

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Protein structures which change their form actively are probably found in all animal and plant cells, at least in certain stages of cell development. These structures have been isolated with retention of their functional capacity from a number of species. Alterations in the form of these structures are the most important cause of biomotility. Only in rare instances may other processes, e.g., changes in osmotic pressure, produce active movements. The causative chemical processes in these cases are unknown.

The movements of structures may be divided into active shortenings and active elongations according to their mechanics; they may also be classified on the basis of the manner in which they are influenced by ATP. Indeed, all movements which are known at present are either produced by ATP or reversed and inhibited by ATP.

If the facts are arranged from these points of view, it becomes apparent on the one hand that entirely different tasks are carried out by the same mechanism and conversely that the same problem can be solved by different mechanisms. The shortening of a muscle and the constriction of a dividing fibroblast have the same mechanism, but the superficially analogous contraction of a muscle and of a *Vorticella* stalk are fundamentally different processes.

I. The Isolation of Motile Structures

Many cell movements, e.g., cell division, are processes of great mechanical complexity. Isolated structures can carry out such movements only if their parts are maintained in the arrangement which they had in life. If these structures are dissolved and floccules are proteins precipitated from the purified protein solutions, these floccules also change their form upon addition of the action substances—they shrink. There is ample proof, at least in muscle, that the reactions of living tissues, of the isolated structures, and of the reprecipitated proteins are basically identical (*1*). Therefore, muscle experiments have been referred to whenever unknown movements and cell proteins were to be investigated.

1. The motile structures* are isolated in insoluble form and their vital arrangement is maintained when the cell membranes are destroyed and the cells and tissues are washed in solutions with an ionic strength $\mu < 0.15$. This is accomplished with concentrated glycerol-water mixtures or with pure aqueous solutions to which cytolytic substances such as saponin have been added. The crystalloids, metabolites, and a large part of the globular proteins then leave the cells. The residual structures are designated as cell models (*2, 3*). They correspond to the so-called muscle models (*4, 5*) and may be subdivided on the basis of their origin into fibroblast models, flagellar models, etc.; or, on the basis of the phase of cell division in which the cells were killed, they may be subdivided into interphase models, anaphase models, and so on.

The method can be applied to single cells. It does not attempt to achieve complete purification of the motile structures, e.g., the removal of the cell granules. It is eminently suitable for the study of the mechanical processes and their conditions; it is unsuitable for the investigation of other activities of the motile proteins, e.g., the splitting of ATP.

2. Many, but not all, proteins of motile structures are dissolved by

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* Motile structures and motile proteins designate those structures and proteins which actively change their form.—The following abbreviations and trade names are used throughout: ATP, adenosinetriphosphate; AMP, adenosinemonophosphate; ITP, inosinetriphosphate; EDTA, ethylenediaminetetraacetate; CTA, cetyltrimethylammonium chloride; Salyrgan, salicyl-hydroxymercuri-methoxypropyl-amidoortho-acetate; Germanin, Na_6-bi-aminobenzene-aminomethyl-benzoylnaphthylaminotrisulfonate.

concentrated salt solutions and are reprecipitated (viz., contractile muscle protein) by dilution. This technique may be combined with differential centrifugation, precipitation by salting out, and other measures, and may be employed for the extraction and purification of the proteins in question. The procedures are more complex with cells than with muscles since cells contain small amounts of motile proteins and many other components of similar solubility.

It is essential to check the purity of each individual preparation carefully. This can be done with the aid of Salyrgan. This mercury compound poisons the contractile ATPases preferentially and leaves the noncontractile ATPases of the granules and cell nuclei largely intact (cf. Fig. 3). The Salyrgan-resistant ATPase activity of the extracts is a measure of their contamination by noncontractile ATPases (*6*).

By the use of methods of this sort, the contractile protein of Jensen and Yoshida sarcoma cells has been isolated (*6*), as well as proteins of slime molds (*7, 8*) and sea urchin eggs (*9*). The latter probably are not contractile but share certain properties with contractile proteins. The motile proteins of flagella, cilia, vorticella stalks, and trichocysts have until now resisted extraction. Statements to the contrary (*10*) are probably based on experimental error (cf. Section IV).

3. A special procedure for the preparation of pure mitotic apparatuses has been reported by Mazia and collaborators (*11, 12*). Up to the present, it has been applied only to sea urchin eggs where a sizable number of synchronously dividing cells can be obtained with ease. The cells are pretreated with 30% alcohol at —10° and the cytoplasm is then dissolved by concentrated digitonin solutions. The mitotic apparatuses remain undissolved and morphologically intact; they can be centrifuged off and dissolved later. However, alcohol destroys the function of the proteins (cf. Section II,C,1).

II. ATP-Driven Single Movements

A. Muscle-like Contractions Driven by ATP Splitting

1. *Rounding Up of Cells*

Not only muscle cells but other cells as well are endowed with active movement. Polymorphous cells round up and retract their processes. The cells round up spontaneously when they get ready for division. Similar movements may be elicited experimentally when amebas are stimulated electrically or mechanically or when substances which cause contractions of muscles (KCl solutions, dilute alcohol, etc.) are added to histiocytes or fibroblasts from subcutaneous tissue (*13*).

All these movements are active contractions. In glycerol-extracted amebas and fibroblasts (models from the amnion fibroblasts and osteoblasts in tissue cultures), they are elicited by ATP; comparative experiments have shown that they resemble the ATP contraction of muscle fibril to the last detail (*3*):

a. Fibroblast models are shortened by the same, very large amount (80%) as muscle models (cf. Fig. 1).

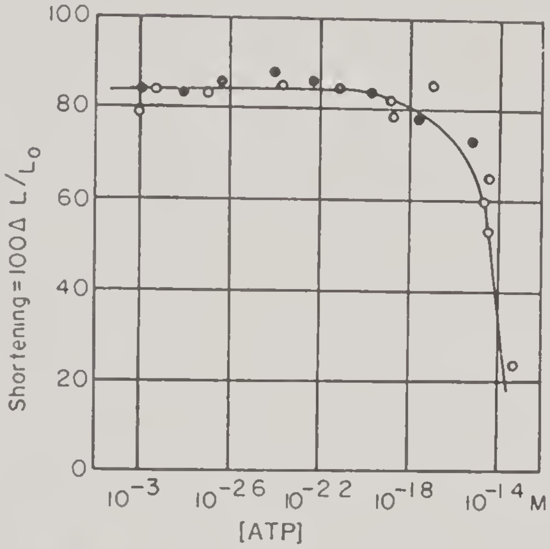

FIG. 1. Dependence of maximal shortening on ATP concentration. KEY: ○, fibroblast models (*3*); ●, fibrils from skeletal muscle (*14*).

b. ATP acts specifically on both types of models. It may be replaced by other nucleoside triphosphates, but not by AMP, creatine phosphate, or other organic phosphates, or by inorganic polyphosphates. Movement ceases if ATP is washed out.

c. Movement takes place only at approximately neutral pH and an ionic strength $\mu < 0.15$.

d. Mg ions must be present. The movements do not occur if all traces of alkaline earths in the preparations are blocked by EDTA. The requirement of the movement for Mg^{2+} is 10^{-1} to 10^{-3} M and 10 to 100 times greater if ITP is used to induce contraction.

e. —SH poisons (e.g., Salyrgan) and certain polysulfonic acids (e.g. Germanin) inhibit the rounding up. The effectiveness of various —SH poisons and polysulfonic acids on cell models is graded in the same way as their effect on muscle models. Poisoning by thiol inhibitors is reversed by cysteine.

f. Cell models also actively develop tension. This may be demonstrated directly if tissue cultures (from Carrel flasks) are treated with glycerol and cut into strips. These strips are attached to a tensiometer in the same way as are muscle models (*6*). ATP evokes tensions up to 22 gm./cm.^{2} (see Fig. 2); muscle models yield up to 4 kg./cm.^{2} The difference is explained by the fact that fibroblasts are poor in contractile protein and that in the strips the cells are connected loosely. They slip away from each other during contraction. In contrast, the tension developed by a single extracted cell is great enough to tear the model to pieces, if shortening is hindered by growing the cultures in a dense fibrin coagulum. Pseudopods of ameba models tear off the cell body.

The strips from glycerol-extracted fibroblast cultures relax like muscle models and their tension drops back to zero if they are poisoned with Salyrgan (Fig. 2).

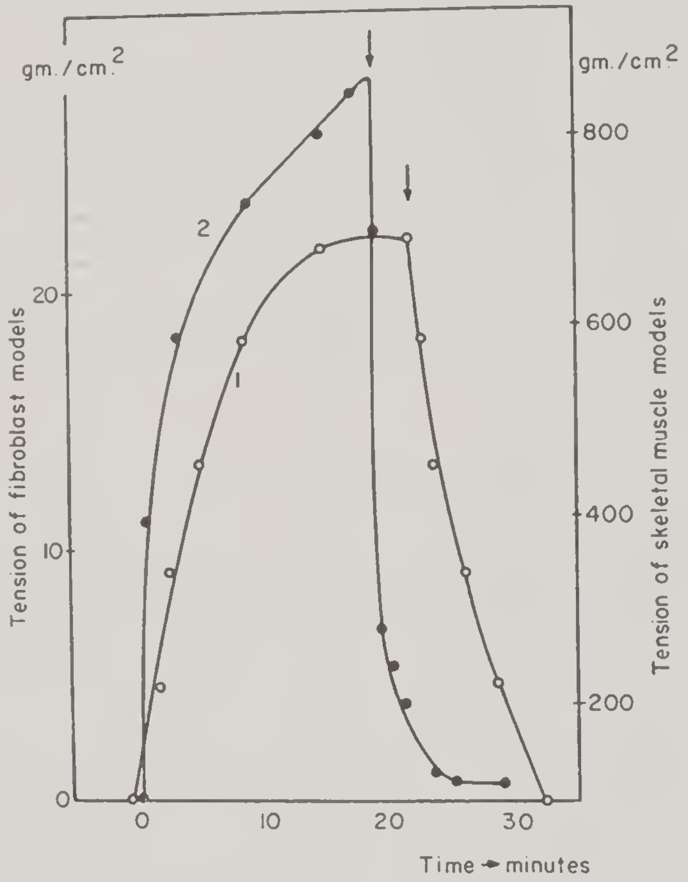

FIG. 2. Development of tension by ATP and relaxation by Salyrgan. Curve 1: (○) strips from glycerol-extracted chick fibroblast cultures (*6*). Curve 2: (●) skeletal muscle models (*123*). ↓, Addition of about 10^{-3} *M* Salyrgan.

Fibroblast models contract much more slowly than muscle models and at the same speed as living fibroblasts [initial shortening at 20° about 0.05% of the relaxation length per second as compared to 200% per second for skeletal muscle models (*14*)]. Cell contraction is slow because its contractile ATP splitting is slow (cf. Section II,B,2).

The close relationship of the movements of a fibroblast and a muscle is shown particularly well by the manner in which both depend on the

ATP concentration. In the wide range between 10^{-4} and 10^{-2} *M* ATP, both fibroblast and muscle models contract maximally and independently of the ATP concentration. If the amount of ATP is increased above this range, they contract more weakly; contractions cease entirely above 2×10^{-2} *M* ATP (*3*). With both types of models, the "supraoptimal" range of ATP concentration is shifted to lower values if the ionic strength is increased. As found in muscle models, contraction is abolished because ATP in high concentrations inhibits contractile ATP splitting (*1*).

These findings elucidate why living fibroblasts round up if their respiration is restricted and why, on the other hand, they fail to exhibit the physiological rounding up at the start of mitosis if ATP is added (*15–17*). Apparently, living interphase cells contain so much ATP that contraction does not occur unless the ATP-resynthesis is reduced.*

Skeletal muscle models relax if treated with ATP plus relaxing factor (Marsh-Bendall factor) (*21*, *22*). The factor prevents ATP splitting at physiological ATP concentrations. It does not prevent splitting and contraction if the ATP concentration is low (*23*). The factor can be extracted from skeletal muscles; for full activity, it requires large quantities of Mg^{2+}(5×10^{-3} *M*). Factor activity is suppressed by 10^{-3} *M* Ca^{2+} (*21*).

Briefly extracted skeletal muscle models still contain appreciable amounts of the factor (*23*, *24*).

Similar observations have been made with fibroblast models. Briefly extracted fibroblasts do not contract if they are placed into solutions whose composition is similar to the ionic environment of living cells: $\mu = 0.2$, $2–5 \times 10^{-3}$ *M* ATP, $5–10 \times 10^{-3}$ *M* Mg^{2+}. They only contract if either the ionic strength or the ATP concentration is decreased or if Ca^{2+} is added. In contrast, cell models and muscle models contract under all conditions if the factor is removed completely by prolonged and exhaustive extraction (*3*).

All in all, it may be stated that systematic comparisons have not yet uncovered any dissimilarity which would indicate a fundamental difference between the contraction of a muscle and the contraction of an undifferentiated cell. The differences are only quantitative. The mechanism of muscle contraction is already present in final form at the developmental stage of the single-cell organism prior to the start of organ formation and cell specialization. It is older than the muscle itself (*25*).

It will be shown later that the cell utilizes its contractility not only for change of form and outward movement but also for other very special tasks (cf. Section II,B).

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* Lettré, however, interprets his findings in a different way (*17–20*).

2. *The Contractile Proteins of Cells and Similar Proteins*

This conformity of cell and muscle contraction is further emphasized by the similarity in properties of the extracted contractile proteins. Studies have been made of the solubility, superprecipitation, ATP splitting, and viscosity behavior of proteins which had been extracted from the Jensen and Yoshida sarcomas of the rat (*6*).

1. These proteins have a similar solubility as actomyosin. At ionic strengths $\mu > 0.3$ they are completely dissolved and at ionic strengths $\mu < 0.15$ they precipitate. In the range of ionic strengths between these two values, ATP promotes the conversion of the gel to the sol. This behavior has been utilized for preparative purposes (Table I).

TABLE I

DEPENDENCE OF SUPERPRECIPITATION AND SOLUBILITY ON IONIC STRENGTH[a]

Ionic strength	Superprecipitation %[b]		Solubility %	
	Contractile sarcoma protein[c]	Actomyosin[d]	Contractile sarcoma protein[c]	Actomyosin[d]
$\mu = 0.06$	100	100	8	0
$\mu = 0.10$	102	80	17	0
$\mu = 0.13$	99	45	24	0
$\mu = 0.20$	49	20	70	40

[a] In the presence of 1×10^{-3} *M* ATP.

[b] Relative density of the superprecipitate in percent of its density at μ 0.06 = 100%.

[c] Hoffmann-Berling (*6*).

[d] Hasselbach *et al.* (*26*).

2. A gel of sarcoma protein shrinks through the action of ATP and superprecipitates when $\mu < 0.15$ (Table I). The colloid chemical alterations are as dependent on temperature and ionic strength as those of muscle actomyosin, they have the same Mg^{2+} requirement ($< 10^{-3}$ *M*) and the same sensitivity to poisons as muscle and cell contraction (Salyrgan, Germanin). The shrinking of the sarcoma protein proceeds 100 to 1000 times more slowly than the shrinking of actomyosin.

3. Contractile sarcoma protein also splits ATP. The splitting is fully activated by 10^{-3} *M* Mg^{2+} (or 2×10^{-2} *M* Mg^{2+} if ITP is split) and inhibited by Salyrgan and Germanin (each at $1\text{–}5 \times 10^{-4}$ *M*) (Fig. 3). These poisons inhibit the contraction of cell models because they block the contractile ATP splitting. The noncontractile ATP splitting of the granules and nuclei is only slightly impaired (Fig. 3). The contractile ATPase of the sarcoma cells splits ATP more than 100 times slower

than does the ATPase of skeletal muscle actomyosin and also more slowly than all other cell structures (granules and nuclei). At 22°, 2.4–3.2 × 10^{-3} μmoles P/mg. protein/minute is liberated.

4. The viscosity of dissolved contractile sarcoma protein is decreased reversibly by ATP and rises once more to the starting value when all the ATP is split (Fig. 4). This may be taken as an indication that the contractile sarcoma protein, like actomyosin, is composed of two protein components which dissociate at high ionic strength and in the presence of ATP.

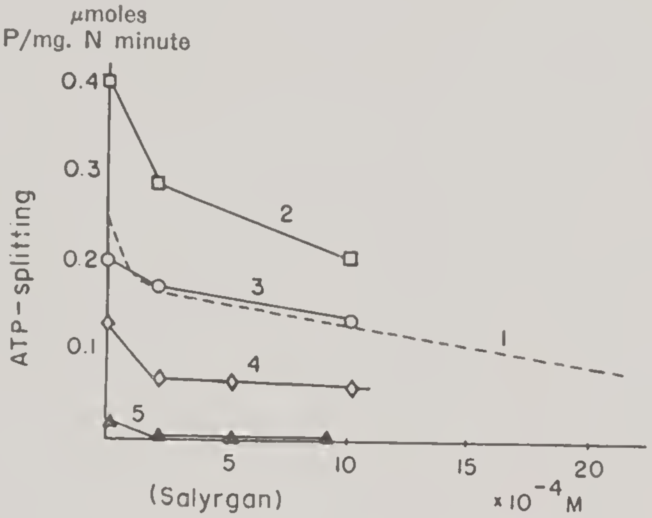

FIG. 3. Inhibition of ATP splitting of fibroblast models and single cell fractions by Salyrgan (*6*). Curve 1: fibroblast models; Curve 2: glycerol-washed cytoplasm of liver cells (without nuclei); Curve 3: glycerol-washed nuclei of liver cells; Curve 4: microsomes of sarcoma cells (isolated in 0.6 *M* KCl); Curve 5: contractile cell protein of sarcoma cells.

Sarcoma cells contain only 0.1–0.2% contractile protein, in contrast to about 10% in skeletal muscle (in terms of fresh weight of tissue). In highly purified preparations, about 5–10% of the ATP splitting are attributable to noncontractile ATPases. This corresponds to a contamination of about 2% microsomal ATPases.

Loewy (*7*) and more recently in particular T'so and collaborators (*8, 27–29*) have extracted a protein from the plasmodia of slime molds (*Physarum polycephalum*) which does not appear to be contractile but which has certain properties in common with contractile proteins. It is known that these organisms move by protoplasmic streaming, and solvation and gelation alternate continuously in their protoplasm. The active protein is called "myxomyosin."

"Myxomyosin" is extracted from frozen plasmodia by 1.4 *M* KCl solution and precipitates between 25 and 36% ammonium sulfate saturation. The purest preparations still contain 20% other proteins. The molecules were shown by electron microscopy to be 4000–5000 Å long, with an axial ratio of 80:1. Their molecular weight is estimated at 6×10^6 based on measurements of sedimentation, electron microscopy, and viscosity.

The protein splits off free phosphate from ATP (calculated at 3.6×10^{-3} μmole P/mg. protein/minute at 24.5°). Therefore, the protein is an ATPase of similar activity as the contractile sarcoma protein. Since the preparations still contain 9% ribonucleic acid, a portion of the activity may be due to noncontractile ATPases.

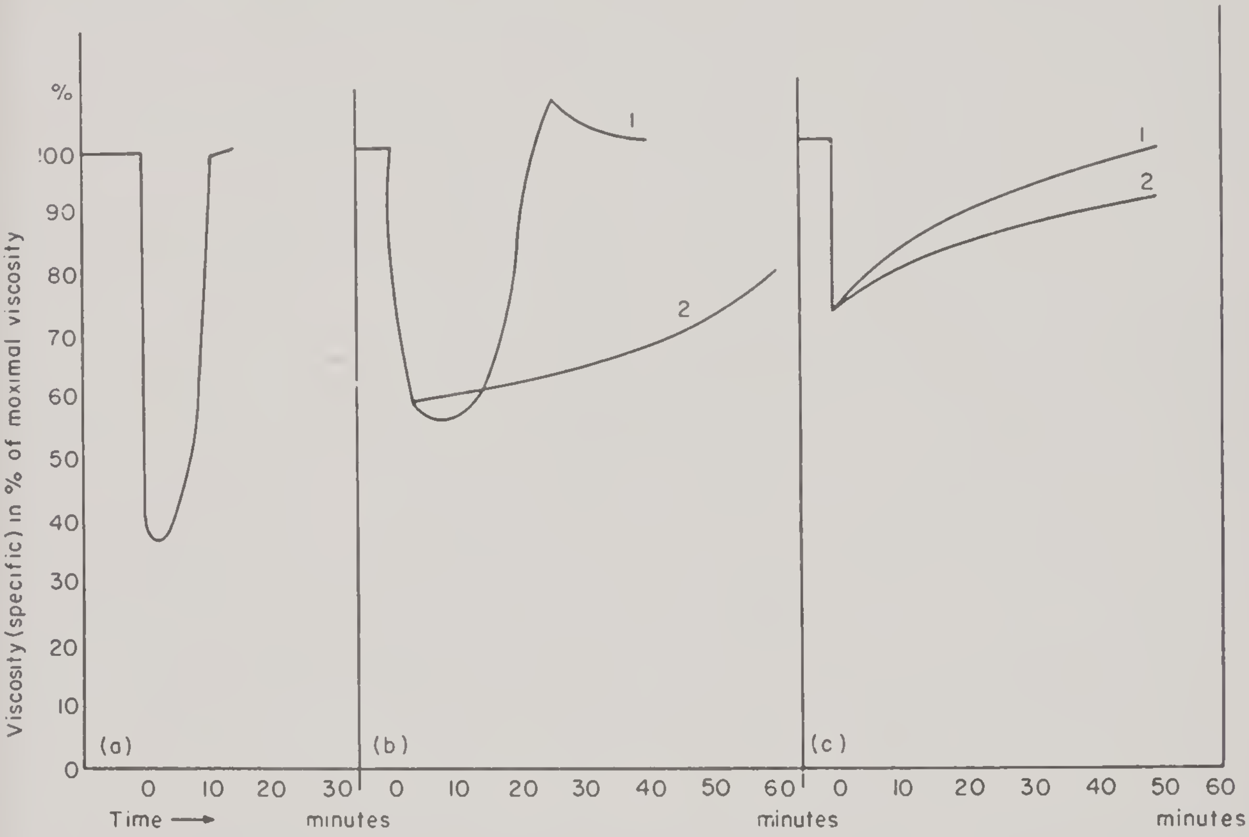

FIG. 4. Alteration of viscosity of different proteins by ATP [sarcoma protein (6), "myxomyosin" (8)]. (*a*) Actomyosin (skeletal muscle), 5×10^{-4} *M* ATP. (*b*) Contractile cell protein (Jensen sarcoma), Curve 1: 2×10^{-4} *M* ATP; Curve 2: 6×10^{-4} *M* ATP. (*c*) "Myxomyosin" (Slime mold), Curve 1: 30×10^{-4} *M* ATP; Curve 2: 3×10^{-4} *M* ATP.

The viscosity of purified solutions is decreased reversibly by ATP, but in contrast to actomyosin and the sarcoma protein this occurs only if the viscosity has first been raised by muscle adenylic acid, phosphate, or by densely packing the protein by centrifugation. After a sharp drop, the viscosity immediately starts to rise again and slowly approaches the starting value (Fig. 4). Added alkaline earth ions have no influence on the course of the curves. According to the authors, the pretreatment brings about an aggregation of the protein molecules which is reversed as long as unsplit ATP is present in the solutions.

The authors find that the cytoplasm of living slime molds is liquefied reversibly by injecting ATP [cf. also Goldacre and Lorch, (*30*)]. They consider this to be a confirmation of their assumption that myxomyosin and similar ATP-sensitive proteins are responsible for the gel state of living protoplasm and that the viscosity changes *in vitro* correspond to the sol-gel transformation *in vivo*. However, there has been no specific proof that myxomyosin forms gels if the ionic strength and pH are lowered to physiological values. Since ATP should be present everywhere in the cytoplasm, even in the solidified portions, there must be additional factors which permit or inhibit, according to need, the liquefaction by ATP.

Although the naming of this protein was intended to suggest relations to the contractile muscle protein, myxomyosin does not appear to be contractile.

In 1936, Mirsky (*9*) extracted a protein from sea urchin eggs which he believed to be myosin-like because it was endowed with structural viscosity and double refraction. These properties were not based on admixed deoxyribonucleoproteins (*31*). However, "myosin-like" proteins extracted from other cells have been identified as deoxyribonucleoproteins (*32, 33*) ["plasmosin" of Bensley and Hoerr (*34*); "renosin" of Banga and Szent-Györgyi (*35*)].

In contrast to muscle, which apparently has firm and relatively unchangeable structures, many other cells continuously rebuild their protoplasm. This was already shown in the example of the slime molds. Marsland and collaborators have attempted with physical methods to clarify by what means this rebuilding is carried out [for a summary cf. Marsland (*36*)]. They find that the protoplasm of many different cells has the tendency to liquefy under pressure (up to 620 kg./cm.^{2}) (*37*). Liquefaction can be followed by stratifying the cell content in centrifugal fields of known gravity. The investigators find a similar drop in viscosity in actomyosin gels exposed to pressure (*37, 38*)]. Marsland therefore classified myosin and living plasma among the gels of type II (Freundlich's classification) which are characterized by the fact that their liquefaction is connected with a decrease in volume and a release of heat. Marsland is therefore inclined to assume that the sol-gel transformation of living protoplasm is a purely physical process dependent on myosin-like proteins (*38*).

B. Elongation Movements Caused by ATP Binding

1. *The Expansion of Rounded Fibroblasts*

A rounded fibroblast can return to the elongated form without a pull from outside—e.g., even if it has been cultivated on a glass substratum in a liquid medium. Seen from the physiological viewpoint (though perhaps not thermodynamically) this movement is an "active" elongation and is reproduced by ATP in the model (*39*).

Models of shortened middle stages of cell division and models of interphase cells which were first shortened by ATP, both elongate "actively." They return approximately to the extended form if they are treated with ATP concentrations which are high enough to prevent contraction. Elongation is particularly pronounced in briefly extracted models which still contain contraction-inhibiting factors.

All measures which inhibit contraction promote elongation: increase of ionic strength, addition of urea [cf. Bozler (*40*)], and so on. It is therefore readily possible to either contract shortened fibroblast models further by addition of small amounts of ATP or to bring them back to the elongated state by the addition of large amounts of ATP.

The differences between elongation and contraction are also shown in other ways. Elongation is not suppressed if the alkaline earths of the preparations are chelated by EDTA. Elongation also fails to be inhibited by the contraction poisons, Salyrgan and Germanin. To an incomplete extent, elongation is even produced by pyrophosphate, which is not split by the contractile structures of the cell. Conversely, elongation is prevented by 10^{-5} gm./ml. protamine sulfate which at this concentration does not interfere with contraction.

Consequently, a separate mechanism is involved which is different from contraction and which presupposes the binding but not the splitting of ATP (at least not by contractile ATPases). It is not known whether the same protein is responsible for the elongation of cells and for their contraction.

2. *The Elongation of the "Stemmkörper"*

The lengthening of the mitotic spindle appears to proceed by the same mechanism as the elongation of the cytoplasm. This movement, which completes the division of the nucleus and falls into the second half of anaphase, is a mechanical process of its own.

Belar (*41*) has shown that the lengthening of the mitotic spindle is an active elongation. He was able to enhance the movement to an extreme by allowing grasshopper spermatocytes to divide in strongly hypertonic solutions. The spindles then coiled up and bent in the cells (*42, 43*).

Spindle elongation is brought about by the inner portions of the spindle. Belar designated these portions as the "Stemmkörper" (pushing body). Inoué (*44*) demonstrated that they are endowed with weak double refraction. When they elongate, they press against the poles of the spindle and the chromosome sets firmly attached to the poles (cf. Section II,C,1) and thus push the chromosome sets further apart.

In fibroblast models from the middle and later stages of anaphase, movement is dependent on the same conditions as the elongation movement of the entire fibroblast (cf. preceding section, II,B,1); however, it is necessary to add urea or to use particularly high supraoptimal ATP concentrations so as to decrease the resistance offered by the cytoplasm (39). ATP and urea "soften" muscle models, i.e., they reduce their resistance to deformation. It may be assumed that they act in this way as well on the cytoplasm of the cell models (Fig. 5).

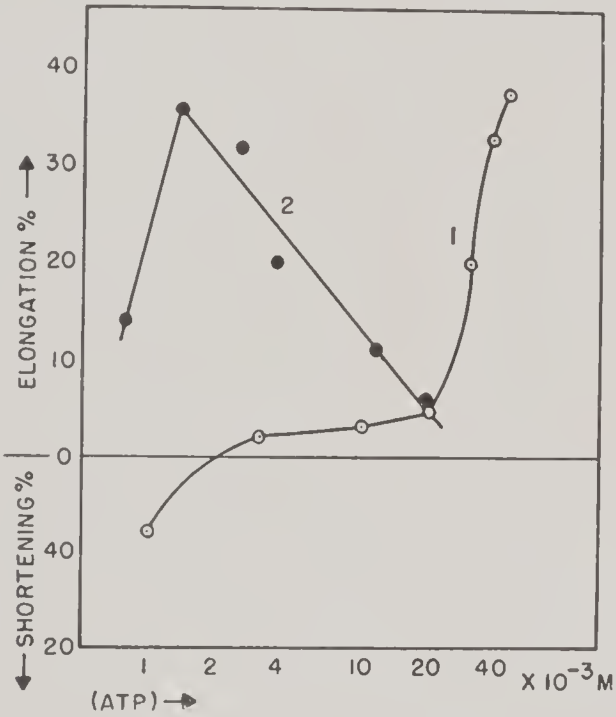

FIG. 5. Elongation of the "Stemmkörper" of fibroblast models in the middle anaphase by ATP (39). Curve 1: (○) without urea; Curve 2: (●) with 0.8 *M* urea.

Colchicine and aminopterin affect neither the elongation of the spindles nor the elongation of the whole models (39).

C. MOVEMENTS BY COMBINATION OF CONTRACTION, ELONGATION, AND OTHER PROCESSES

1. *The Movements of the Spindle Apparatus in Anaphase and the Purified Spindle Proteins*

No other cell movement has been studied as frequently and explained in so many different ways as the separation of the chromosomes in anaphase* (46). From the abundance of theories, those may be eliminated which either deny or ignore the existence of the mitotic spindle. It is implausible that an organelle should not have a role in division if it is formed especially for the division, if its cohesion with the chromosomes is proven by isolation, if its form changes with the movement of the chromosomes, and if a disturbance in or failure of its formation makes division impossible.

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* For a critical review see F. Schrader (45).

In the opinion of this reviewer, the greatest credence should be given to concepts which postulate not one, but two mechanisms of chromosome movement, such as the theory developed by Ris (*47*) [cf. Hughes and Swann (*48*); Jacquez and Biesele (*49*)]. According to Ris, the chromosomes are transported in sequence by traction and pressure. The theory has the advantage of having been confirmed at least in part by direct experiments.

Each chromosome is firmly connected with the proper spindle pole by contractile fibers (chromosome fibers). Together with the chromosomes, these form the outer portions of the spindle. The poles of the spindle, in turn, are held away from each other by the spindle contents —which are identical with the "Stemmkörper" of Belar (*41*). The poles become fixed points for the contraction of the chromosome fibers which draw the chromosomes close to the spindle poles.

The counter force of this movement is the "Stemmkörper." It becomes the active part in the second half of the anaphase. The elongation of the "Stemmkörper" pushes the spindle poles further apart, with the adhering chromosome fibers and chromosomes, and into the future daughter cells. The two movements follow each other in time and are complementary. In different cells, they probably contribute to division in varying degrees. There are extreme instances where only the shortening of the fibers or only the elongation of the spindle is observed.

The following arguments support this theory:

a. The chromosome fibers are visible in fixed cells and are indicated in living cells by a positive double refraction of the spindle (*44, 50, 51*). The double refraction is directed toward the two spindle poles. As the chromosomes move toward the poles, the double refraction decreases. Schmidt (*52*) already concluded from this that the fibers contract, because the double refraction of muscle fibers also decreases when they contract [cf. also Cornman (*53*)].

b. In the beginning of anaphase, just before the chromosomes start to move, the spindle transiently becomes short and broad (*44, 48, 54*). This is what should be expected, if the chromosome fibers first contract isometrically and compress the spindle.

c. In fibroblast models prepared from cells in early anaphase, the chromosomes occasionally have been observed to move apart under conditions which activate contractile mechanisms but exclude spindle elongation (*39*).

Attempts to poison muscles and living mitoses with the same substances [cf. Hughes (*46*)], and thus to make a parallel between the contraction of the spindle fibers and muscle contraction, have been unsuccessful. The mitotic poison colchicine, but also the less specific mitotic poison aminopterin as well as monoiodoacetamide and chloral hydrate, fail to act on the contraction of muscle models (*55*). Findings of a slight alteration in the reactions of dissolved actomyosin caused by colchicine (*56*) are not mirrored by the behavior of the models. It may be assumed that all these poisons interfere with the processes of synthesis or energy production but not with the mechanical processes. How damaging colchicine is for the construction of the spindle is demonstrated by the deformed mitotic apparatuses isolated from colchicine-poisoned egg cells (*12*).

d. Whereas the contraction of the chromosome fibers is still open to discussion, the second portion of anaphase, the active elongation of the spindle (or of the "Stemmkörper") (*39, 42*) has been proven (cf. Section II,B,2).

It is to be expected that the contractile mechanism and the mechanism for elongation of the spindle do not act simultaneously but rather alternate as the motor of chromosome movement. Indeed, the conditions of contraction and of active elongation are on the whole mutually exclusive (see Section II,B,1, but also cf. II,C,2).

If really two mechanisms are united in the spindle, then certain peculiarities of the mitotic apparatus are easily explained: the mitotic organelle is small, though chromosomes are to be transported from zero distance over a path which is longer than the initial spindle length. The entire apparatus is mechanically independent of its environment and its functioning is not disturbed if it is displaced within the cell or if the cell is deformed.

It must be mentioned that very different concepts have been developed about the contraction of the chromosome fibers. According to Swann (*51*) contraction is initiated by a substance which is excreted by the chromosomes and which diffuses toward the poles of the spindle. This concept is derived from alterations in double refraction. Chemical investigations have not been made.

It is self-evident that the energy metabolism of a cell does not tell us anything about the mechanism of its division. The finding of an increased requirement for nutrients (*57*), increased heat production (*58*), or heightened respiratory activity—which Zeuthen has demonstrated with certainty for the middle stages of division by the use of refined techniques (*59*)—does not permit us to conclude that it is just the mechanical processes which have an unusually high energy requirement. It is not known how the extra metabolism is distributed to the various actions of the cell. It is probable that even very motile cells use only a small portion of their energy for mechanical work (*60*). Moreover, there are cells which still can carry out one or more divisions even after complete removal of oxygen or poisoning of respiration (*57*).

Mazia and collaborators (*11, 12, 61*) have isolated the nuclear mitotic apparatus of sea urchin eggs. The mitotic apparatus is composed of chromosomes, spindle, and polar radiation (cf. Section I). After poisoning with colchicine, the mitotic apparatus is missing or is very incompletely formed (*12*). From synchronously dividing eggs it is possible to isolate mitotic apparatus in amounts sufficient for analysis.

The isolated mitotic apparatus represent 2–12% of the total cell protein. They are soluble in 0.5 *N* NaOH, alkaline thioglycolate, and concentrated solutions of urea or rhodanide, but not in concentrated salt solutions. The spindle protein, therefore, is presumed to be widely different from the contractile muscle proteins. In all these data, however, it must be kept in mind that the proteins of the spindle have certainly been changed by the pretreatment with 30% alcohol. Actomyosin also loses its solubility after pretreatment with alcohol.

The protein dissolved in alkaline thioglycolate migrates in two fractions in an electrical field. One is a nucleoprotein and probably is derived from the chromosomes. The other appears to be the spindle protein itself. Its amino acid composition is not dissimilar from that of native actomyosin (1 part actin, 3 parts myosin). From the amino acid analysis, a minimum molecular weight of 22,000 is calculated (*12*).

The spindle protein is viscous and has double refraction of flow when it is dissolved in concentrated urea solutions, and it loses these properties when it is treated with thioglycolate. The protein molecules are thought to be connected lengthwise by S—S bridges and the bridges are broken by thioglycolate. From this idea, a theory of spindle formation is derived (*12*).

Mitotic apparatuses isolated according to the procedure of Mazia do not split ATP and also fail to move.

2. *Cytokinesis*

The constriction of the cell equator (cytokinesis) by which animal cells divide has the appearance of a contraction (*62–64*), but this explanation is not generally recognized. Earlier investigators sought the cause in forces of surface tension and emphasized that oil drops also constrict when their surface tension is lowered symmetrically (*65*).

Among more recent investigators, Rashevsky and his biomathematical school also consider cytokinesis a movement of a fluid phase (*66*). A concentration gradient of unknown substances are thought to cause currents in the cell which lead to elongation and "instability." It is postulated that surface forces bring about division secondarily. Equations are derived for elongation and constriction whose agreement with the measured values of increase in length (not as much those of constriction) are adduced as support for the theory (*67*).

The following opposing evidence [cited after Hughes (*46*)] seems of significance: egg cells divide regularly even if portions of the cytoplasm are removed and the symmetrical concentration gradient postulated by Rashevsky is disturbed (*68*).

Mitchison and Swann* consider the cell membrane to be the active part, at least in animal egg cells. However, the cell membrane is thought to cleave the cell not because of its surface tension but by an active expansion in area (*70–74*). During this proccss, the membrane folds inward ("expanding membrane theory").

The theory is supported by arguments which have no direct bearing on its central point, i.e., the *active* expansion of the membrane. Probably there are no ways at all at present to prove that a membrane is expanding actively (*75*).

There are good reasons to suppose that it is not the cell membrane, but cytoplasmic structures, which are the motor of cytokinesis. This is supported by experiments in which dividing cells were liquefied by high pressures. Division stops in all cells under these conditions. Constriction even regresses if the cytoplasm of the division furrow was included in the liquefaction area (*36, 76, 77, 78, 79, 80*).

In fibroblasts, the cytoplasmic structures are arranged especially densely and they are unusually resistant to experimental manipulation. Glycerol-extracted fibroblast models from the late stages of nuclear division cleave if ATP is added. No other substance has this effect. ATP induces constriction under conditions similar to those which induce rounding up of the interphase models: for instance, Mg^{2+} is required; the movement is inhibited by the specific poisons of cell and muscle contraction (*81*) (cf. Section II,A,1).

Cytokinesis of the models—like cytokinesis of living fibroblasts—is no simple contraction. A more detailed analysis showed that the models elongate lengthwise at the same time. Mechanically this elongation is necessary for the proper formation of the equatorial contraction. The simultaneous course of the two movements is strictly dependent on the presence of physiological ATP concentrations.

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* For a review see Swann and Mitchison (*69*).

At higher ATP concentrations, the contraction of the cleavage furrow fails to take place although the model still elongates. A constriction which has already begun can even be reversed. Conversely, ATP in low concentrations will allow constriction to start. But the models also contract at the cell poles. Thus material is pushed into the middle of the cell so that the division furrow is expanded. Such cells finally round up without cytokinesis.

As the data show the same, physiological ATP concentration causes contraction in the middle of the cell but relaxation at the cell poles. The poles are more sensitive to the contraction-inhibiting effect of ATP than are the equatorial portions of the cell. These differences are prerequisites for division and are explained most simply if the assumption is made that the models still contain contraction-inhibiting factors which are not evenly distributed [as in interphase models (*3*)] but are more active or more abundant in the cell poles. The uneven distribution is preserved for some time in the models.

This explanation is supported by two facts:

a. Upon longer extraction, all differences disappear. Thoroughly washed telophase models do not cleave but round up.

b. Addition of Ca^{2+} which blocks relaxing factor activity, has the same effect (*81*) (cf. Section II,A,1).

The dissolution of the cytoplasmic strand which still connects the two daughter cells after maximal contraction presents a problem of its own. Only when this problem is solved will the analysis of cytokinesis be complete.

3. *The Locomotion of Amebas*

According to Lewis (*82*), the crawling movement of an ameba is a combination of three processes: (*a*) cortical protoplasm contracts at the posterior end and pushes the liquid cell contents forwards; (*b*) the contracted protoplasm liquefies and becomes involved in the transport movement; (*c*) the liquid plasma is channeled in such a way through the middle of the cell that its stream bends like a fountain at the anterior end. As soon as the protoplasm comes in contact with the cell surface it solidifies again.

The cause of locomotion, then, is a contraction. The cell contents are moved passively by its pressure.

That the inner cell contents are liquid and the outer protoplasm is solid can be demonstrated by a series of independent methods, e.g., by centrifugation of the amebas: the inclusions which lie in the inner portion of the cell are displaced easily, the inclusions in the cell cortex are displaced practically not at all (*83*).

That the entire cytoplasm of the cell is involved in the cycle of liquefaction and solidification is shown by the observation of single cell inclusions and their thermic movements (*84*).

That the plasm of amebas is contractile is proved by model experiments (*6*). Models are not free of artifacts. They contract not only at the posterior end but as a whole. Glycerol extraction activates the contraction even of those sections of the cells which are liquid *in vivo.*

Goldacre and Lorch (*30*) found that amebas take up basic dyes from the surrounding medium and store them at their tail. This phenomenon was observed only when the cells were streaming.

These authors suggest that a contractile particle folds when it shortens and that its active surface then becomes smaller. Then relaxed particles should store more dye than contracted ones. The authors refer to globular proteins which when unfolded by denaturation bind more dye than in the folded native state. In the authors' opinion the storage of dye is the result of contractile folding in which the contractile proteins liberate adsorbed dye (*85*).

Experiments with contractile muscle protein fail to confirm this hypothesis. The ability of actomyosin to adsorb dyes is completely independent of its contraction state (*86*).

Furthermore, Goldacre and Lorch (*30*) find that the cytoplasmic streaming of a living ameba is accelerated reversibly if ATP is injected into its posterior end and that the direction of streaming can be reversed if ATP is injected into the anterior end. A special analysis of these findings is hardly possible because ATP effects are strongly dependent on ATP concentration and ionic conditions (cf. Section II,A).

The processes of contraction and reversible liquefaction which are thought to lend motility to an ameba have thus been demonstrated separately. Whether they cooperate in the manner postulated by the theory remains an open question.

III. Movements Which Are Inhibited or Made Reversible by ATP

The multiplicity and variety of the movements which depend on ATP as the direct energy source, led to the impression that a reaction with ATP was the only way in which motile structures could be activated. This generalization cannot be maintained. There are active contractions as well as active elongations which are induced by ATP-free reactions. These special forms of motility have been encountered only in protozoa. They are now described in some detail because they provide an interesting contrast to the ATP-driven movements.

A. THE CONTRACTION OF *Vorticella* STALKS

Certain ciliates (*Vorticella*) form a motile stalk which carries the cell and with whose aid the animal attaches itself. The stalk contains a contractile element, the "myonema" (*87*), in a flexible sheath. If the organism is irritated the contraction of the myonema bends the sheath into a tight spiral; because of the elasticity of the sheath, the stalk straightens out again when the myonema relaxes. The process resembles a flight reaction but may also occur spontaneously or as a spontaneous rhythm (*88*).

Stalks and muscles have various properties in common. A myonema contains microscopically visible fibrils (*87, 88*). The myonema exhibits positive double refraction. The latter decreases in a contraction (*89, 90*). Contraction can easily be induced by electrical stimulation (*91*) and its speed of shortening is as high as that of a cross-striated muscle.

1. *ATP-Free Contraction and Relaxation*

On the other hand, stalks and muscles differ in their contractile mechanisms: models of *Vorticella* stalks do not contract under any conditions if treated with ATP; conversely, they contract promptly when exposed to Ca^{2+} or Sr^{2+} ions or ions of quaternary ammonium bases (*91a, 92*). Tension developed during contraction is so great that the contractile elements may even tear themselves. Organic compounds, e.g., cetyltrimethylammonium salts (CTA) must carry a large nonpolar molecular residue in addition to a single positive charge, as the so-called invert soaps do. Mg^{2+} and Be^{2+} cause no contraction, Ba^{2+} only a weak one.

Contraction is reversed if bound Ca^{2+} is removed by EDTA; the stalks relax and elongate again. The contraction caused by CTA is irreversible.

By alternating exposure to Ca^{2+} and EDTA, contraction and relaxation can be repeated as often as desired. The contractions, therefore, are not elicited by traces of ATP retained by the models. Such traces should be used up completely by this continuous performance of work. Moreover, the Ca^{2+}-contraction of the stalks is insensitive to the ATPase poisons Salyrgan and Germanin.

Since the models lack all metabolic and excitatory processes, the contraction producing agents must act directly on the contractile protein. All effective substances are cations which carry positive charges into the contractile system. Electrostatic alteration of the protein seems to be the immediate cause of contraction. The fact that not all cations are effective must be explained by specificities of binding. Contraction caused by alterations of the charge have been predicted by Wöhlisch (*93*) and Meyer (*94, 95*) and analyzed by experimentation with polyacrylic acid fibers (*96, 97, 98*).

The physiological agent among the various cations probably is Ca^{2+}. It is certainly present in living systems, acts at the lowest concentration, develops the highest speed of contraction, and its action is reversible at will.

2. *The Rhythmical Relaxation Caused by ATP*

The only known physiological substance which causes relaxation is ATP. Its action differs fundamentally from that of EDTA: ATP does not cause the models to relax permanently but induces a rhythm of spontaneous relaxations and contractions (*92*).

The capacity of ATP to complex Ca^{2+} (*99*) is not sufficient to explain these effects, for the following reasons:

a. Calculations show that under suitable conditions a much larger concentration of Ca^{2+} ions remains unbound in ATP solutions than in citrate or oxalate solutions. The latter two substances do not have a relaxing effect.

b. ATP cannot be replaced by the related ITP although ITP has the same affinity for Ca^{2+} as ATP (*100*).

c. The relaxing action of ATP increases with the Mg^{2+} concentration up to about 5×10^{-3} M Mg^{2+}. This would not be understandable if ATP were to act only as a complexing agent. Since Mg^{2+} is more efficiently bound by ATP than Ca^{2+} (*99*), a large excess of Mg^{2+} should liberate practically all the Ca^{2+} from its ATP binding.

d. ATP does not lead to permanent relaxation. Rather, the relaxation is interrupted rhythmically.

e. The relaxing action of ATP is prevented by Salyrgan, whereas this drug does not affect the relaxation caused by EDTA (cf. Section III,A,1).

All these findings show that physiological relaxation occurs because ATP reacts with the contractile protein and not because it binds the Ca^{2+} ions present in the solutions.

Since in model systems no other source of energy is available for a continuous rhythm than ATP splitting, the contractile *Vorticella* protein should split ATP. For technical reasons, this has not yet been demonstrated by measurement. But the paralyzing action of Salyrgan is an argument in favor of this assumption. To abolish the rhythmical behavior of the stalks, the same low concentrations (5×10^{-5} M) of Salyrgan suffice as are required for the poisoning of the ATPases of muscles and sarcoma cells. At these concentrations of Salyrgan, other ATPases remain intact (5) (cf. Section II,A,2).

The movement of the *Vorticella* stalks is made complex primarily by the course of events during relaxation. Physiological relaxation is not simply the reverse of contraction. The chemical energy of ATP is utilized by means of a complicated process in the relaxation phase and is stored in the contractile system.

B. THE ELONGATION OF TRICHOCYSTS

Trichocysts are structures in the outer layers of certain ciliates and flagellates which are ejected upon stimulation or even spontaneously. As they shoot off they elongate to 6–7 times their original length and push themselves away from the cell. Their volume also increases to about the same extent. The function of the trichocysts is unknown; they have been considered a vehicle for the excretion of salts (*101*).

The trichocysts of paramecia are composed of a tip and a shaft which is the organ of movement. The elongated shaft may split up into fibrils (*102*), it is endowed with double refraction, and this double refraction is composed of a positive form and a positive intrinsic birefringence. The shaft may be hollow and exhibits a characteristic cross-striation in the electron microscope. Paramecia may be kept from shooting off their trichocysts by Ca^{2+}-binding agents, e.g., EDTA. If such cells are broken, the trichocysts can be concentrated and purified by differential centrifugation (*103*).

1. *ATP-Independant Elongation*

Isolated trichocysts of *P. caudatum* elongate explosively and by the same amount as living trichocysts when Ca^{2+} or Sr^{2+} or the ions of invert soaps, e.g., CTA (cf. Section II,A,1), are added, but not upon the addition of Mg^{2+}. There is no evidence for a capsule from which the structures are liberated.

The movement can be followed visually if it is slowed by pretreatment with protamine sulfate. Then it becomes apparent that it always begins at the tip, i.e., the peripheral portion of the trichocyst, and proceeds along the shaft of the trichocyst. Every section which becomes involved in the movement promptly elongates to the maximal possible extent.

2. *The Inhibition of Elongation by ATP*

Although the movements of the trichocysts and of the vorticella stalks differ in their mechanics and in their ion requirements, they resemble each other in that they are inhibited by ATP. The Ca^{2+}-induced elongation of the trichocysts is prevented if ATP is present. Naturally, a completed elongation is not reversed.

In this instance, too, the inhibitory action of ATP is not related to its binding of Ca^{2+} ions. The presence of 10^{-3} *M* ATP prevents the action of almost twice this quantity of Ca^{2+} ions. Since the solutions still contain Mg^{2+}, which is bound preferentially, and since there are no Ca-ATP complexes with more than one Ca atom in the complex (*99*), almost 10^{-3} *M* Ca^{2+} ions should still remain unbound. But this is a concentration which more than suffices to induce ATP-free trichocysts to shoot off.

If the trichocysts after ATP treatment are also poisoned with Salyrgan ($5–20 \times 10^{-5}$ *M*), the inhibition of the shooting off is maintained even if ATP and Salyrgan are washed out. On the other hand, the same concentrations of Salyrgan do not inhibit elongation if they are used without ATP. The Ca^{2+}-induced elongation of trichocysts which have not come in contact with ATP is inhibited only by much higher (10^{-3} *M*) concentrations of Salyrgan.

In trichocysts it can be demonstrated directly that Salyrgan acts as an enzyme poison. Trichocysts contain an ATPase which at 22° liberates about 0.03 μmole P/1 mg. protein × minute from ATP and which is 70% inactivated if it is treated with $5–10 \times 10^{-4}$ *M* Salyrgan (the possibility cannot be excluded that a part of the Salyrgan-resistant activity is referable to impurities).

In trichocysts, similar speculations as in *Vorticella* stalks may be advanced. In both cases, the inhibitory activity of ATP could arise from an intermediary product of a chain of ATP-protein reactions. The inhibition of the Ca^{2+}-induced elongation in this instance would be maintained even after removal of ATP if the destruction of the intermediate is blocked by Salyrgan (cf. Section III,A,2).

IV. The Rhythmical Movements of Flagella and Cilia

Mere observation of living flagella and cilia shows that they move actively and that their form is altered by processes which take place within them and not in the underlying cells: their undulating movements can continue undiminished from the base to the tip of the flagella; the tip may even develop a higher angular velocity than the base so that the flagella bend against the resistance of the medium. This is possible only if energy is fed in not only at the site of insertion but over the whole length of the flagellum (Gray, *104*).

It is generally true that flagella beat only in one plane, but some of them can also execute helical movements. The flagella of sea urchin spermatozoa, which have been investigated thoroughly, carry out two-dimensional movements. The amplitude of their beat is 4 μ, the frequency of their movement 30–40 beats/second [determined stroboscopically (*105*)], the velocity with which the curvature travels along the axis of the flagellum 800 μ/second. The flagellum shortens on the concave side by about 5% with a speed of 15% of its length per second (*106*). According to Gray, the simplest assumption is that unsymmetrically acting contractile elements distort elastic components of the flagellum.

However, a clear-cut separation into contractile and elastic elements is not found in electron microscope pictures. Only the flagella of mammalian spermatozoa seem to be an exception. They contain a spiral which encloses the fibrils and which is thought to be elastic and not contractile (*107*). Beside this, the electron microscope has uncovered the fact that practically all animal (*108*) and plant (*109*) flagella and cilia are built according to the same plan: 9 outer fibrils (in the flagella of mammalian spermatozoa 2×9) surround two narrow inner ones. The inner fibrils are destroyed easily and were overlooked at first. The direction of the beat of the flagellum is perpendicular to the plane which can be drawn through the inner fibrils. It has been assumed that they are not contractile but that they serve as conductors of stimuli and coordinate the activity of the outer contractile fibrils (*107*).

It is hard to say why the arrangement of $9 + 2$ fibrils is so advantageous that it is retained essentially unaltered. Moreover, it is rather unexpected that the same arrangement is used for two-dimensional as well as three-dimensional movements. The mechanics of two-dimensional movements are easily deduced if groups of fibrils facing each other contract alternately.

This simple assumption is not sufficient to explain helical movements. For a contractile cylinder to carry out such movements, at least these formal conditions must be fulfilled according to Reichert (*109*): the sites of simultaneous contraction must be located in such a way that they can be connected by a helical line on the surface of the cylinder, and this line must be shifted parallel to itself. It is easy to see that these conditions would be fulfilled if the outer fibrils were to contract one after the other and each over its entire length and if they were coiled around each other. But it is exactly this arrangement which is missing. To satisfy the conditions of Reichert, it must rather be assumed that contraction waves migrate on the single fibrils and that the waves on contiguous fibrils are shifted by a constant amount. This could take place by an excitation wave which circulates in the basal apparatus of the flagellum and reaches the fibrils in the sequence of their insertion. The contraction waves of neighboring fibrils, then, would be shifted in relation to each other by the amount of time required by the stimulus to jump from one fibril to the next (*107*).

The basal apparatus, then, would be the center of automation of the movement. In fact, it has been observed that as a rule flagella detached from their cells remain motionless if they have lost their basal apparatus.

The flagella of bacteria do not fit into the organizational scheme. They are narrow (diameter of 130 Å) and have room for only a few peptide chains; for this reason it is highly improbable that they are simply a miniature copy of the flagella of more highly organized cells. Moreover, they appear to be constructed in the form of a helix, for they form helical aggregates when they are precipitated by salting out (*110*).

Since bacterial flagella are easily separated and obtained pure, they have become a favorite object for analysis (*111–113*). The flagella of *Proteus vulgaris, Salmonella paratyphi,* and *Bacillus subtilis* form highly viscous solutions endowed with double refraction of flow. Upon slight acidification they are broken up irreversibly into split products of uniform size. The molecular weight of the split products has been determined by ultracentrifugation and diffusion measurement to be 41,000. The axial ratio is 15:1.

Bacterial flagella consist almost entirely of protein (16% N). They do not contain measurable amounts of purines and only 1% carbohydrate, probably as an impurity. Pijper (*114*) is incorrect in claiming that bacterial flagella are accidentally formed aggregates of the polysaccharides of the bacterial capsules and that they are without significance for the motility of their carriers (*113*).

One property, however, is shared by bacterial flagella and the flagellar protein of higher cells and organized actomyosin systems: as Astbury has shown, they all belong to the same k-m-e-f group of fibrillar proteins (*110, 115–117*).

The motility of bacteria is abolished by thiol inhibitors (organic mercury compounds, monoiodoacetate) and restored by cysteine (*118*). At least in monoiodoacetate poisoning, a block of the sensitive energy-yielding metabolism appears to be involved.

Alterations of the flagellar structure (*119*) reported to be induced by ATP appear doubtful because the light scattering and colloidal behavior of suspensions of flagella (from *B. proteus*) are not altered by ATP. But such a change is to be expected if the flagella change their form. Occasionally, ATP was slowly split by the suspension of flagella (*120*).

Animal flagella are better suited than bacterial flagella for investigations concerned with function because with the former a single flagellum can be followed. Glycerol-extracted models from the unusually large spermatozoan flagella of tropical locusts (*Tachycines*) or from *Trypanosoma brucei* (from the blood of infected mice) regain the motility of living cells as soon as ATP is added. The models from *Tachycines* flagella continue to move even if their basal apparatus is detached and the flagellum is dissociated into several strands by weakly alkaline solutions (*121*). Models have also been prepared from the flagella of mammalian spermatozoa, but certain additional precautions are necessary if motility is to be maintained (addition of colloids etc.) (*122*).

ATP induces optimal rhythmical activity in the models only if Mg^{2+} is added also. EDTA suppresses motility, which may be restored by Mg^{2+} but not by Ca^{2+}. A concentration around 10^{-3} *M* Mg^{2+} is optimal.

SH-poisons paralyze the models and their sensitivity to the various poisons of this type is graduated like that of muscle models. Salyrgan again is most effective whereas monoiodoacetate is almost noninhibitory. Germanin and related polysulfonic acids also poison flagellar motility. A particular sensitivity to Germanin is exhibited by trypanosome models. It may be recalled that Germanin was developed for the treatment of trypanosomal infections.

The agreement between flagellar and muscle models is further extended by the finding that for flagella too there is a limit of ATP concentration above which their motility ceases. For *Tachycines* flagella this limit of ATP concentration is about the same as for muscle models (2×10^{-2} *M* ATP). With both test objects it is lowered by high ionic strength. Trypanosome models are still more sensitive to ATP than spermatozoan flagella.

The findings leave little room for doubt that ATP is the energy source of flagellar movement. In this regard it acts specifically and cannot be replaced by other organic phosphates (with the probable exception of nucleoside triphosphates) and inorganic polyphosphates.

But in addition ATP determines the frequency of flagellar movement. And in this it acts nonspecifically. In the range of ATP concentrations between 10^{-5} and 10^{-2} *M* ATP the frequency of the flagellar beat increases with the logarithm of the ATP concentration. But a movement of high frequency also results if inorganic pyrophosphate or urea is added or if the ionic strength of the solutions is increased, provided that the minimum ATP concentration is maintained which is required to cover the energy requirement of the movement (about 6×10^{-6} *M* ATP) (Fig. 6). Since all these additions plasticize muscle models, i.e., lower their resistance to stretch (*123*), higher ATP concentrations apparently accelerate flagellar movement because deformability determines whether the flagellar beat is rare or frequent. In muscle models also, the specific action of ATP is accompanied by a nonspecific softening effect.

In the glycerol-extracted pharyngeal mucous membrane of the frog it has been shown as well that ATP restores the ciliary beat (*124*).

That ATP causes the rhythm of the flagellar beat does not mean that ATP produces the contraction phase of the movements. ATP could just as well transfer its energy into the relaxation phase, as was the case in the *Vorticella* stalks. Indeed, it is impossible to tell by looking at the nonmoving flagellum whether it is in a totally contracted or relaxed state. Consequently, it cannot be decided whether ATP produces the flagellar rhythm by inducing originally relaxed structures to contract or by inducing originally contracted structures to relax. If previously the muscle-like interpretation was preferred, it was because at that time the effect of ATP on Ca^{2+}-contracted systems was not recognized and it was not known that certain Ca^{2+}-induced contractions become rhythmical through the action of ATP.

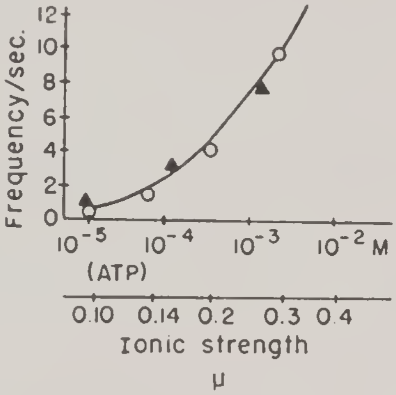

FIG. 6. Change in the beat frequency of flagellar models [from spermatozoa of *Tachycines* (*121*)]: *a.* (▲) by increasing ATP concentration at constant ionic strength ($I = 0.20\mu$), *b.* (○) by increasing ionic strength at constant ATP concentration ($[ATP] = 5 \times 10^{-4} M$).

A choice could probably be made between these various possible explanations if the contractile protein of the flagella could be extracted. Unfortunately, it has resisted the usual extraction procedures almost completely.

Flagellar protein appears to be soluble only under extreme conditions, e.g., in alkaline solutions, which rapidly destroy the function of other contractile proteins. Protracted treatment with such solutions or with distilled water do liberate components which hydrolyze ATP (*10*). But the extracted ATPases cannot be poisoned by Salyrgan (*122*), which is deleterious to the rhythmical movements of the flagella models. On the other hand flagella (particularly in mammalian spermatozoa) contain granules along almost their entire length which presumably are the seat of extractable ATP-splitting enzymes (*107*).

V. Summary

Four processes have been discussed by which the structures of muscles and cells convert chemical into mechanical energy. Two result in shortening; the other two in elongation. In all the processes ATP is involved, either as an initiator of motility or as an inhibitor and relaxing agent. The conditions on which the action of ATP depends have certain features in common, but interpretations of the underlying processes are necessarily indirect, since the isolation and characterization of cell proteins in most cases is made difficult by the small quantity of starting material and the low solubility of the structures.

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CHAPTER 7

Active Transport

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I. Definition

It is characteristic of living cells that the composition of the cell interior is different from that of the surrounding medium, not only with respect to large molecules like proteins but also with respect to most small molecules and ions. The cell must take up and excrete matter in order to maintain the proper conditions for the processes of life. The differences in the composition of the internal and external media therefore cannot be due to the presence of an impermeable membrane. Likewise in plants and cold-blooded aquatic animals, the internal medium is different from the external, despite the fact that at least part of the body surface is permeable to some substances in the surrounding medium. Even on the subcellular level differences in composition exist. Thus the particulate cell constituents maintain an internal medium different from the cytoplasm, although they exchange matter with the cytoplasm.

In the last decades the problem of how these differences are created and maintained has become the subject of intensive studies. It was revealed that although the movement and distribution of many substances could be accounted for by the action of purely physical forces, such as those arising from concentration gradients and electric potential gradients, in some cases these forces did not suffice. Therefore the concept of active transport was introduced. At the present time we are still left with the same negative definition of active transport: a substance can be regarded as actively transported only if the transfer of the substance across a membrane cannot be accounted for by the action of the forces of diffusion, electric potential gradient, solvent drag, or these forces in any combination.

This definition includes not only the cases where a substance is moved against its electrochemical gradient but also the cases where a substance is transferred along the gradient faster than would be expected from the magnitude of the above mentioned forces.

If a substance is transferred against its electrochemical potential gradient, this means that its free energy is increased during the transport process. This extra energy can be obtained from one source only, i.e., metabolism. At present little is known about the details of the coupling between metabolism and transport processes. As more knowledge is gained about this coupling, the requirement of a simple stoichiometric relation between the amount of substance transported and the amount of metabolites consumed will become of more importance in testing mechanisms proposed for active transport. Energy is also spent when a substance is transported along the potential gradient. But in this case it may be difficult to relate the amount of substance transported to the amount of energy used because it is not always possible to determine the active part of the total transfer.

Other definitions have been advanced, but none are unequivocal. Thus among kidney physiologists a maximum transfer rate (T_m) for a substance is often taken as evidence for active transport. Although active transport mechanisms do have a limited transport capacity the same can be true for passive transfer, for instance when binding to a specific substance in the membrane is essential for the transfer. As an example, the transfer of glucose across the red blood cell membrane may be mentioned. Although the rate of transfer at higher glucose concentrations becomes independent of concentration, the transfer process eventually leads to equilibrium across the membrane [see, for example, Willbrandt (*1*)]. Dependence upon metabolic activity has also been used as a criterion for active transport. However, this definition will also classify osmotic water transfer as being active if the difference in osmotic pressure across the membrane depends on metabolism. The definition nearest to the one advanced here uses transfer against the electrochemical potential gradient as the criterion for active transport (2). It does not, however, take solvent drag into account.

II. Experimental Characterization

To be able to decide whether a given substance is actively transported, one must know how a substance which is not being transported actively behaves under the influence of concentration gradient, electric potential gradient, and solvent drag. The latter occurs only if bulk flow of solution can take place through the membrane, i.e., if the membrane is porous. If this is the case, electroosmosis or a difference in pressure (hydrostatic or osmotic) between the two solutions bathing the membrane will cause net flow of solvent. Solute particles diffusing in the direction of flow will be speeded up, whereas particles diffusing in the opposite direction will be slowed down. Generally the drag effect is of little or no importance, but it may under suitable conditions become considerable. A relation between the flux of a substance (unidirectional flow per unit area per unit time) and the above-mentioned three forces can be obtained, but the equation contains so many unknowns that a general solution cannot be given. However, most of the unknowns cancel out in the expression for the flux ratio, i.e., the flux of substance going from one side of the membrane to the other divided by the flux in the opposite direction. The equation then is:

$$\ln M_{12}/M_{21} = \ln a_1/a_2 + (zF/RT)(\psi_1 - \psi_2) + (1/D) \int_0^{x_0} (1/g'_w)(dP/dx)dx \tag{1}$$

M_{12} and M_{21} designate the two fluxes as measured with isotopes; a_1 and a_2, the chemical activities of the substance in the two bathing solutions; z, the charge of the substance (positive, negative, or zero); F, the Faraday constant; R, the gas constant; T, the absolute temperature; $\psi_1 - \psi_2$, the electric potential difference across the membrane; D, the free diffusion coefficient in water; g'_w, the friction between one mole of water and the membrane at unit velocity; dP/dx, the force driving water through the membrane; x, the distance in the membrane from one of the boundaries; and x_0, the thickness of the membrane. (For a detailed treatment, see references 3–5.) If the pore population is not homogenous, but composed of groups of pores each of a fairly uniform pore size, Eq. 1 has to be applied to each group separately.

The applicability of Eq. 1 has certain limitations. In the first place, a general solution of the integral cannot be given, since both g'_w and dP/dx may vary in an unknown way with x. However, it will be noted that the value of the integral for a given membrane under constant conditions is independent of the nature of the solute, which may even be water itself. Therefore if the value of the last term in Eq. 1 has been determined for one substance which is not being actively transported, the drag effect on another substance may be calculated. Because of the way in which the drag effect has been evaluated, there is one obvious limitation to this approach. This is that the substance for which the calculation is made must penetrate the membrane by exactly the same pathways as does the substance by which the integral has been determined. The cell membrane is probably composed of hydrophilic and lyophilic areas arranged in a mosaic. If a substance is lipoid-soluble to any extent, part of it will cross the membrane dissolved in the lipoid part of the membrane, and thus cause an underestimation of the solvent drag. It is important to realize that solvent drag is due to the friction between water and solute. Hence the free diffusion coefficient, D, is the proper parameter to use, and not the permeability coefficient, which is a measure of the solute-membrane interaction. The correctness of using D depends on the assumption that the viscosity of the solution is practically unchanged in the pore. Thus far, evidence in conflict with this assumption has not been found.

As to the experimental characterization of active transport, we may first consider the case in which no net transfer of water occurs. In this case Eq. 1 reduces to

$$\ln M_{12}/M_{21} = \ln a_1/a_2 + (zF/RT)(\psi_1 - \psi_2) \qquad (2)$$

Since this equation contains relatively easily measurable parameters, experimental conditions are preferably chosen to allow the application of this equation. When epithelial membranes are dealt with, the chemical activities and the electric potential difference $(\psi_1 - \psi_2)$ across the membrane can readily be measured. The fluxes can be measured by suitable experiments with the use of isotopes. It is thus easy to determine whether or not a given substance behaves in accordance with Eq. 2.

When membranes on the cellular or subcellular level are dealt with, the situation becomes somewhat more difficult. In many cases the chemical activity of a substance cannot be determined in the internal medium. However, it can safely be assumed that the activity coefficient for any substance is at most unity. Therefore, if the concentration is determined, it can be used as the upper limit for the chemical activity. Information about the electric potential gradient across the membrane is generally unobtainable, especially when we deal with subcellular membranes. In some cases the potential difference across cell membranes can be measured directly with internal microelectrodes. In some other cases an estimate may be made from the distribution of ions that can be assumed to behave passively. In these cases, where at least a fair guess can be made about the magnitude of the right-hand side of Eq. 2, the only safe procedure is to test whether or not a net transfer can take place against the electrochemical potential gradient.

Sometimes one has to reckon with a passive flow of water through a membrane. Under these conditions the last term in Eq. 1 becomes important. As mentioned above, if the integral has been determined for one (passively behaving) substance, the drag effect can be calculated for any other substance which penetrates the membrane through the same pathways. As an example we may consider the case where water itself is the "solute." Equation 1 then reduces to:

$$\ln M_{12}/M_{21} + \ln a_1/a_2 + (1/D_w) \int_0^{x_0} (1/g'_w)(dP/dx)dx \qquad (3)$$

where D_w is the self-diffusion coefficient of water. Whereas the linear rate of flow, $-(1/g'_w)(dP/dx)$, may vary with x, the volume rate must be independent of x. The volume flow rate, Δ_w $(= M_{12} - M_{21})$, is equal to $A(1/g'_w)(dP/dx)$ the area available to flow times the linear flow rate. Hence, $(1/g'_w)(dP/dx) = \Delta_w/A$, which inserted into Eq. 3 yields:

$$\ln M_{12}/M_{21} = \ln a_1/a_2 + (1/D_w) \int_0^{x_0} (\Delta_w/A)dx$$

or, since Δ_w is constant,

$$\ln M_{12}/M_{21} = \ln a_1/a_2 + (\Delta_w/D_w) \int_0^{x_0} (1/A)dx$$

By simultaneous determination of Δ_w $(= M_{12} - M_{21})$ and one flux with heavy water, the integral can be evaluated.

It has to be pointed out that in general solvent drag is of minor importance in comparison with gradients of concentration and electric potential. However, it cannot be ignored when a considerable transfer of water takes place, as, for instance, across the intestinal wall or across the kidney tubule wall.

If a substance behaves in accordance with Eq. 1, it can be concluded that the substance is not being actively transported through the membrane. On the other hand, a disagreement with Eq. 1 does not as such constitute proof of active transport. Equation 1 is derived with the assumption that the substance under investigation is not chemically bound to any moving particle during passage through the membrane, and that molecules or ions move independently of other molecules or ions of the same species. If these requirements are not fulfilled, one cannot in general expect an agreement between the actual behavior of a substance and the behavior predicted by Eq. 1. Experimental observations have been taken as evidence for three ways in which a passively behaving substance may pass a membrane, viz., by free diffusion, by exchange diffusion, and by single file diffusion. As already mentioned, Eq. 1 is strictly valid only for the first-mentioned possibility.

Exchange diffusion may occur under certain conditions if a substance permeates a membrane in combination with another substance. Let us consider the following case. A substance, *A*, combines specifically with another substance, *X*, in the membrane. *X* is unable to diffuse out of the membrane and is neither created nor destroyed at the two membrane boundaries. Furthermore neither *A* nor *X* can cross the membrane separately, but only as parts of the complex *AX*. Due to thermal agitation complex molecules will move back and forth from one boundary to the other. When in contact with one of the bathing solutions, the complex may exchange the bound *A* for another *A* from the solution. As neither *A* nor *X* can diffuse alone, no net transfer will occur whether the fluxes as measured with isotopes are high or low. The work performed is of course zero. The transfer may depend on metabolic energy for synthesis of the carrier, *X*, but no stoichiometric relation will exist between the amounts of metabolites used and the amount of substance transferred. It is well to remember that even if *A*, *X*, or both are able to cross the membrane in other forms than *AX*, exchange diffusion may still play a role in the transfer. As mentioned below, this possibility has to be taken into account when considering the energetics of active transport.

The concept of 'single file' diffusion has been introduced in order to explain the following phenomenon. Across the fiber membrane of axons poisoned with dinitrophenol in order to stop active transport, potassium ions move according to the equation $M_{\text{in}}/M_{\text{out}} = (\bar{a}_{\text{out}}/\bar{a}_{\text{in}})^n$, where n has values between 2 and 3, as if the potassium ion had a charge of 2 or 3 (*6*). Since this cannot be the case, it has been proposed that 2 or 3 ions move as a unit in the membrane. The most likely way in which this can happen is that the ions pass through channels where 2 or 3 ions are lined up at any time. The ions are supposed to be unable to pass each other; thus if one ion enters the membrane from one side, another ion has to leave at the other. Both statistical considerations and model experiments show that such an arrangement will explain the observed behavior, but it should be emphasized that alternative mechanisms are not excluded.

As has been made clear, Eq. 1 may not always be used to decide whether a substance is actively transported. There is, however, one important set of conditions for which the equation can be used unequivocally to settle the problem, i.e., when $\bar{a}_1 = \bar{a}_2$. Under these conditions there will be a net transfer of only those substances which undergo active transport. In many cases, however, these conditions cannot be obtained because the medium on one side of the membrane is inaccessible for control.

III. Proposed Mechanisms

Thus far nothing has been said about the underlying mechanisms of active transport. In the following paragraphs some of the hypotheses proposed for the processes responsible for active transport will be discussed.

The most commonly accepted theory, the simple membrane-carrier theory (7), postulates the existence in the membrane of a substance with which the actively transported substance specifically combines. If the complex can diffuse across the membrane, active transport can be brought about by maintaining a concentration gradient for the complex in the direction of transport. This can be done by a continuous synthesis of the carrier at one boundary and a consumption of the carrier at the other boundary. The sequence of events would thus be: synthesis of carrier, combination with partner substance, diffusion of the complex across the membrane, and dissociation of the complex by consumption of the carrier. Another possibility is that the carrier is converted at one boundary into a substance which is unable to combine with the partner substance, but which, after returning to the other boundary, is reconverted into the active form. In both cases work has to be performed by the cell in order to maintain the concentration gradient. It will be noted that the mechanism for exchange diffusion suggested above differs from the mechanism for active transport outlined here only in that there is no gradient for the complex in the membrane. In fact, cases are known where a substance traverses a membrane partly by way of active transport and partly by way of exchange diffusion.

Electron-linked transport has been proposed both for anion and cation transport (8, 9). The mechanism would consist in a transference of electrons, via a chain of respiratory enzymes of the hemin group, from a boundary where the oxydation power is low to a boundary where the oxidation power is high. The Fe of the enzyme will thus alternately have a valency of two or three. Anion transport could take place in the following way. Since trivalent Fe attracts one more anion than does divalent Fe, the transfer of an electron from one link of the chain to the next will cause an anion to move in the opposite direction. The basic reactions taking place at the two terminals of the chain are:

$$4\ Fe^{3+} + 4\ H \rightleftarrows 4\ Fe^{2+} + 4\ H^+$$

and

$$4\ Fe^{2+} + 4\ H^+ + O_2 \rightleftarrows 4\ Fe^{3+} + 2\ H_2O$$

This means that in reality electrons and hydrogen ions would be the true objects of active transport. The hydrogen atoms in the first reaction are provided by substrates for dehydrogenases. Cation transport is supposed to be accomplished by an exchange of hydrogen ions for the cations in question. The hypothesis shares with other hypotheses based on electric forces the difficulty of explaining the high degree of specificity shown by the transport mechanisms. In the redox pump hypothesis (*9*), the difficulty is overcome by postulating the participation of a carrier driven by the transfer of electrons across the membrane.

It will be noticed that according to both hypotheses of electron-linked transport, not more than four equivalents of ion can be transported per molecule of oxygen consumed. In plant tissue, ratios up to almost six have been reported (*10*), but the authors themselves conclude from mean values for the ion to oxygen ratio that the value of four was not exceeded in their experiments. It is not appropriate, however, to use mean values for determination of maximum efficiency, unless the experimental errors are greater than the biological variation. The ratio of four monovalent ions per molecule of oxygen is definitely too small in the case of sodium transport in the isolated frog skin (*11*). Even if the total amount of oxygen consumed by the skin is assumed to be used for the transport of sodium, ratios higher than four are often found. The hypothesis has been modified to yield a higher maximum ratio (*12*); but the limiting ratio still seems to be smaller than those found for the active transport of sodium ions in the frog skin.

The fluid circuit hypothesis has been proposed for the uptake of salt from the small intestine of the dog (*13*). In this hypothesis it is assumed that the membrane (intestinal wall) contains two sets of pores. Through one set of pores a salt solution passes the membrane. Through the other set of pores, water, or at least a less concentrated solution, is returned. The force responsible for the back flow of water is thought to be electroosmosis. In this hypothesis one is again faced with the difficulty of explaining the observed specificity of the transport processes.

The two last hypotheses which will be mentioned both propose structural changes in macromolecules (proteins or conjugated proteins) as the basis for active transport. In the first hypothesis the basic mechanism is an alternation between the extended and contracted (coiled) state of a contractile protein molecule (*14, 15*). The process of uncoiling involves breaking of intramolecular bonds, thus forming affinities which can only be satisfied by adsorption of foreign molecules or ions. When the protein molecule is recoiled the original bonds are reformed and the foreign matter is desorbed. The protein can either be fastened at one end, the other end moving back and forth across the membrane, or the protein can diffuse across the membrane. It is suggested that the contractile molecule possesses adenosine triphosphatase activity, resembling the actomyosin complex. The second hypothesis involves a change in the lattice of a macromolecule (*16*). When the lattice expands at one membrane boundary, molecules or ions of the right kind can move into the lattice. If the expansion proceeds by a kind of peristaltic wave in the lattice, the molecules or ions can be transported across the membrane.

IV. Types of Active Transport

In general the active transport processes are highly—though not absolutely—specific. In many cases a group of closely related substances seems to be transported by the same mechanism, although not necessarily with the same efficiency. In some other cases even substances that are chemically rather different seem to compete for the same transport mechanism. Before some of the types of active transport are dealt with, it must be mentioned that only in relatively few cases has unequivocal proof been given for the active nature of the transport process. In other cases, although conclusive proof is missing, there is good reason to assume active transport.

In animal cells (and for that matter in nearly all cells) the sodium concentration is generally low and the potassium concentration is high, as compared with the concentrations in the extracellular fluid. In neurons (*17*) and in muscle cells (*18*) it is practically certain that the low sodium concentration is due to an active extrusion of sodium ions. In these cells a net extrusion can take place against both a concentration gradient and an electric potential gradient. The potential difference across the red blood cell membrane has not been measured; but from the distribution of chloride (and bicarbonate) ions, which are believed to behave passively, the interior of the cell is considered as being slightly negative (less than 10 mv.) relative to the external medium. Thus sodium ions are also actively transported out of the cell in this case (*19, 20*). There is little reason to doubt that the same mechanism is at work in many other cells as well. As the assumed magnitude of the potential difference across the red blood cell membrane is far too low to account for the high internal potassium concentration, it is readily realized that potassium ions also undergo active transport (*19, 21*). In muscle and nerve, on the other hand, the potential difference is sufficiently high to account for the internal concentration of potassium. However, evidence is accumulating which indicates that potassium is also being transported actively in muscle and nerve fibers, in the direction opposite to that of sodium. It is interesting that sodium and potassium transports across cellular membranes seem to be coupled (*22, 23*), so that, in effect, the active sodium transport is a "forced exchange" of sodium against potassium. (For a recent review see reference *23a.*)

Sodium is often transported actively across epithelial membranes. Among these the frog skin has been studied most extensively. The skin is able to transport sodium chloride from the outside solution to the inside. By the "short circuit" technique (for explanation see Fig. 1) it has been shown that under normal conditions only sodium ions undergo active transport, while chloride ions are passively dragged along by electric attraction (*24*). As there is no detectable active transport of potassium across the frog skin, sodium ions may seem to be transported by different transport mechanisms across cellular and epithelial membranes. The epithelial cells of the frog skin are, however, rich in potassium and

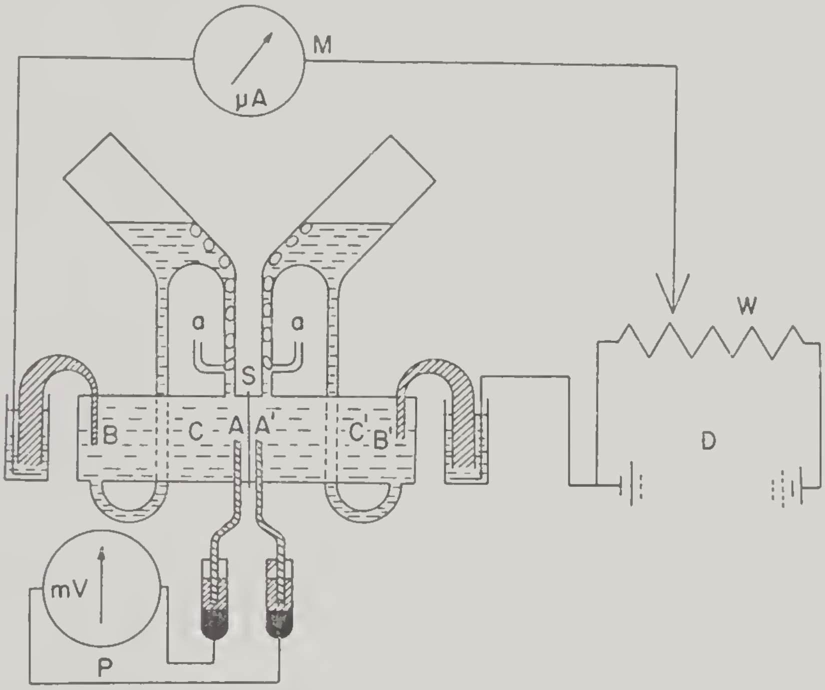


FIG. 1. Diagram of apparatus used for determination of ion fluxes and short-circuit current. The membrane, *S*, is placed between two chambers, *C*. The bathing solutions are circulated and gassed by air entering through the inlets, *a*. Two agar Ringer bridges, *A* and *A'*, connect the solutions with two calomel electrodes. The potential difference across the membrane is measured by means of a potentiometer, *P*. This potential difference can be maintained at zero by application of the proper voltage from the battery, *D*, through the agar bridges, *B* and *B'*. The current flowing in the circuit is measured with a microammeter, *M*. The two fluxes of any particular substance can be measured either by the use of two different isotopic tracers, say Na^{22} and Na^{24}, or by determination of the flux in one direction and the net flux. If the electrochemical activity of a substance is the same on both sides of the membrane, the net flux should be zero unless the substance is actively transported.

probably possess some mechanism for maintaining this composition. Thus they may have the same "ion pump" as the other cells, i.e., an active potassium transport linked with the active sodium transport (see below). The assumption is supported by the observation, among others, that cardiac glycosides have similar effects on active sodium transport in muscle, red blood cells, and frog skin. This point will be dealt with again below; here the discussion will deal in particular with how the operation of a sodium-potassium exchange pump may give rise to a one-sided transport of sodium.

The small intestine of the rat has the ability to transport sodium as well as chloride ions in the same direction, i.e., from the mucosal (luminal) to the serosal side (*24a*), a phenomenon that has not been observed in other tissues as yet. This dual transport system explains the surprisingly low spontaneous potential across the intestinal wall.

It has long been known that sodium chloride is reabsorbed from the tubular fluid. By micropuncture of single nephrons it has been shown that, in the proximal tubule, salt is taken up, together with enough water to maintain approximate isotonicity of the tubular fluid (*25*). An early attempt to measure the potential difference across the tubular wall in amphibia led to the assumption that the electric force was of little importance (*26*). Recently the problem has been reinvestigated in rat kidney (*27*). It was found that in different proximal tubules the potential difference ranged from about 20 to 40 mv., the lumen being negative. The range in distal tubules was from 30 to 70 mv., the lumen again being negative. This clearly shows that sodium is actively transported. There is at present no need to assume active transport of chloride. This is in agreement with the assumptions based on excretion studies.

Lately it has also been shown that a considerable transtubular potential (about 20 mv.) exists across the proximal tubules in amphibia (*27a,b*). The disagreement with the earlier investigation very likely is due to the use of much thinner microelectrodes, thus avoiding damage of the tubule and local shunting of the potential.

The urine can under certain conditions contain more acid than has been filtered through the glomeruli. Since pH values as low as 4.6 may be found, passive excretion of hydrogen ions would require the lumen to be up to 180 mv. negative relative to the fluid around the tubule. Such a high potential is unlikely, although not impossible. It is more likely that the acidification is brought about by an exchange of hydrogen ions against sodium ions. A sufficient supply of hydrogen ions could most easily be obtained from carbonic acid through the action of carbonic anhydrase. If so, inhibitors of the enzyme should inhibit both sodium reabsorption and acidification. In fact, these effects are observed in acidotic dogs to which inhibitors of carbonic anhydrase have been administered (*28, 29*). At the same time potassium may be excreted with a higher clearance than inulin. (This is not as such proof for active transport.) If mercurials are then administered, sodium ions are excreted at a still higher rate, whereas the excretion of potassium ceases. These observations are consistent with the view that hydrogen ions are excreted in exchange for sodium ions, but if the supply of hydrogen ions becomes inadequate, potassium ions can substitute. It is possible that potassium ions and sodium ions are normal partners. Micropuncture experiments on amphibian kidney indicate that almost all potassium is reabsorbed in the proximal tubule, whereupon excess potassium is excreted in the distal tubule (*30*).

Of the other alkali metal ions, only lithium can substitute for sodium in some cases. Rubidium and, to a certain degree, cesium behave like potassium.

The potassium transport in yeast may constitute an example of active cation transport in plants. Potassium is taken up at the onset of fermentation in exchange for hydrogen ions (*31, 32*). At the end of fermentation potassium is given off again. If potassium is absent from the external medium, hydrogen will be excreted largely in the form of succinic acid. The transport mechanism is very specific and will accept other ions only when sufficient amounts of potassium are lacking. Sodium apparently does not participate in the exchange reaction, which thus clearly differs from the above mentioned exchanges in animal cells. It has, however, been claimed that besides this exchange of hydrogen ions for potassium ions there is another exchange reaction by which yeast cells enriched in sodium ions will excrete the sodium in exchange for potassium ions (*32*).

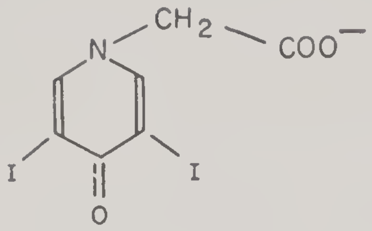
Recently two algal species, a characean *Nitellopsis obtusa* (*32a*) and a large single-celled alga *Halicystis ovalis* (*32b*) have been used to study active transport in plants. These organisms are particularly suitable for the purpose because the cells are large and the vacuoles contain ample sap for analysis. In the case of *Halicystis ovalis* it has even been possible to change the vacuolar content and to short-circuit the cell. In these two organisms active transport of both chloride and sodium ions has been demonstrated. The chloride ions are transported into the vacuole while the sodium ions are extruded from the cell. There is some evidence that the two transport mechanisms are spatially separated, the chloride transport taking place in the protoplasm-vacuolar sap interphase (the tonoplast) and the sodium transport presumably at the protoplasm-external medium interphase.

In higher plants much more work has been done on uptake of salts by roots than on transport across membranes in other parts of the plants. Two different kinds of preparation have been used, viz., excised roots from plants of the grass family, and thin disks of tissue from roots used as storage organs. The phenomena studied in excised roots are transcellular transfers of salts from the external solution across one or several cell layers into the fluid in the central vessel. In experiments with disks the transfer involves penetration of only one cell membrane (and the vacuolar membrane) and thus results in an accumulation of salts in the cell sap. Although the concentrations of some salts may vary in very much the same way in cell sap and in fluid from the central vessel (bleeding sap) when the external medium is changed (*33*), the two transport mechanisms need not be of the same type. In both kinds of preparation used, salts can be taken up from very dilute solutions. Whether the uptake is caused by an active transport of anions only (*8*) or whether cations are also actively transported (*34, 35*) is still a matter of dispute. In wheat root the bleeding sap seems to be sufficiently negative relative to the external solution to account for at least the better part of the potassium uptake (*36*). The potential difference and the potassium concentration were, however, not measured in the same experiment. It is not known whether the potential difference between cell sap and outside medium is large enough to account for the accumulations of cations. The anion transport mechanisms seem to exhibit a low degree of specificity. Not only will the closely related halogen ions be taken up, but also the nitrate and sulphate ions will be transported. However, the efficiency with which the ions are taken up may be quite different. As an example the accumulation of halogen ions by carrot disks may be mentioned (*10*). When the increase in oxygen consumption of disks transferred from distilled water to solutions of different salts having equal concentrations is compared with the concomitant uptake of salt, it is found that the number of ions taken up per mole of excess oxygen consumed is largest for chloride and smallest for iodide.

A low specificity of chloride transport seems to be the general rule in animals also. Thus the gastric mucosa will transport not only chloride (*37*), but bromide (*38*) (even better) and iodide as well (*39*). Likewise, the thyroid transports chloride and bromide (*40*), although to a lesser degree than iodide (*41, 42*). In this case there is good evidence that the different kinds of anion really are transported by the same mechanism. If excess chloride or bromide is fed to rats the iodine content of the thyroid will decrease and the animals will acquire goiter. Furthermore, under propylthiouracil poisoning the thyrotropic hormone can be shown to increase the ability of the gland to concentrate all three anions (*43*). (Propylthiouracil is used in order to prevent the formation of organic iodine compounds).

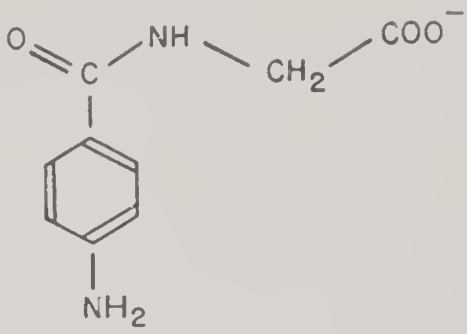
Due to the tendency of phosphate ions to form easily dissociable complexes, especially with proteins, determination of the activity of phosphate ions in biological fluids may be very difficult. The matter is further complicated by the existence of two biologically important kinds of phosphate ion, of which both or just one may permeate. The investigation of phosphate transfer across the cell membrane of a bacterium, *Staphylococcus aureus*, may serve as an example of how these difficulties may be overcome (*44*). It was observed that the apparent intracellular phosphate concentration could be maintained at a level one hundred times higher than that in the external solution. If the cell interior was strongly positive relative to the outside, the combined effect of complex formation and electric attraction might account for the uneven distribution, especially if phosphate permeated as the divalent ion. However, since lysine is passively concentrated in the cell whereas glutamic acid appears to be actively transported into the cell (*45, 46*), it seems safe to assume that the cell interior is negative relative to the outer medium. Since it is unreasonable to assume that less than one per cent of the intracellular inorganic phosphate is uncomplexed, there can be little doubt that phosphate ions are actively transported by this microorganism.

Transport of organic substances has been studied most extensively in the kidney. Among the organic anions several groups are actively secreted into the proximal tubular lumen. One group comprises the iodinated compounds, exemplified by Diodrast (iodopyracet), used for X-ray examination of the urinary system (*47*). The iodine-free nuclei are excreted as well, showing that iodine is not essential in the transport process. Another group is formed by hippuric acid and its derivatives, of which the most well known is *p*-aminohippuric acid (PAH) (*48*). Further, several sulfonated dye-stuffs, such as phenol red (*49*), and the penicillins (*50*) are actively secreted. All these chemically quite different compounds (I–IV) compete for the same mechanism. The only thing they seem to have in common is the simultaneous occurence of strongly hydrophilic and lipophilic groups within the molecule. At least in PAH the unmodified carboxyl group is essential for the transport processes (*51*). PAH labeled with oxygen-18 in the carboxyl group does not lose the label during transport; this fact demonstrates that the transport processes do not involve breaking of the C—O bond. This rules out that certain types of esters, for instance thiol esters, are formed between carrier and PAH, or that peptide bonds are formed. Formation of a compound of the type —COO—PO_3HR is still possible.



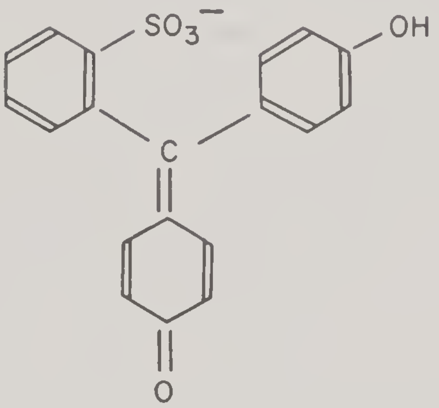
DIODRAST

I



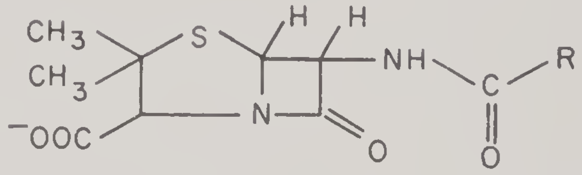
p-AMINOHIPPURATE

II



PHENOL RED

III



PENICILLIN

IV

An example of amino acid transport in a bacterium has already been mentioned. As another example, the active reabsorption of amino acids in the proximal tubules may be considered. The amino acids apparently form three groups. Within a group, competition takes place, but not between groups and neither does glucose absorption seem to interfer with the transport of any amino acid (52). One group comprises the basic amino acids histidine, lysine, and arginine. Leucine and *iso*-leucine form another group (53). The third group is formed by glycine, alanine, glutamic acid, and creatine (52). In all cases the specificity seems to be absolute as far as the stereochemical configuration is concerned. D-Amino acids are excreted almost exclusively from a racemic mixture (54, 55).

Among the nonelectrolytes notably glucose and certain other carbohydrates have been studied with respect to their active transport. Glucose is found to be actively absorbed both in the intestine (56) and in the proximal tubules (*57*). The two transport mechanisms apparently are not of the same type. Thus glucose and other carbohydrates, e.g., xylose, probably vie for the same transport mechanism in the proximal tubule, since high loads of glucose block the transfer of xylose (*58*). In the intestine, xylose behaves passively and the transfer is not affected by glucose loading (*59*).

A very interesting attempt to relate steric configuration of glucose derivatives with ability to undergo active transport in the gut has recently been published (*59a*). Starting with the observation that 3-*O*-methyl-D-glucose is actively taken up by the gut (*59b*) other derivatives of glucose were tried. Apparently a D-pyranose structure and a hydroxyl group in the glucose configuration at carbon-2 are essential for active transport. On the other hand, glucose can be modified in the groups in position 1, 3, and 6 without loosing the ability to be actively transported. This shows that if phosphorylation is an essential step in the transport processes the esters cannot be of the usual 1- or 6-glucose phosphate type.

Finally the transport of gases into the swimbladder of fishes shall be mentioned. It has been known for some time that some species of deep sea fishes may have oxygen at a pressure of more than one hundred atmospheres in the swimbladder. It has been suggested that the oxygen was derived from some metabolic processes, but with the aid of oxygen-18 it has been demonstrated that the cod derives all the oxygen from molecular oxygen (*60*). In some other species (not deep sea), for instance in the whitefish, the gas in the swimbladder is mainly a mixture of nitrogen and argon. The proportion between the two gases is the same in the swimbladder as it is in the surrounding water, which is in equilibrium with the atmosphere (*61*). This proves that the gases cannot be derived from metabolic processes in this species either. It has been claimed that accumulation of gases is brought about by a countercurrent multiplying system (*rete mirabile*). Since some species do not possess a *rete mirabile,* but nevertheless are able to deposite gas against a steep pressure gradient, this explanation obviously is not satisfactory (*62*). Since argon is concentrated as much as is nitrogen, the two gases most likely are transported by the same mechanism. This mechanism cannot very well be of the simple carrier type, but may rather be of the expanding lattice type mentioned above (*16*).

It has, however, lately been found that in some species with a *rete mirabile* the ratio between argon (or helium) and nitrogen in the freshly accumulated gas mixture may be different from that of the gas dissolved in the external medium. A hypothesis has been suggested in which the mechanism of gas accumulation in the swimbladder consists in formation in the *rete mirabile* of small bubbles of oxygen into which the other gases diffuse. After entering the swimbladder, oxygen is selectively removed from the gas mixture (*62a*).

Whereas there is general agreement that solutes can be actively transported, divergent opinions exist about the possibility of active transport of water. Fundamentally, transport of any compound of a solution will influence the behavior of any other component. (For a theoretical treatment see reference *63*). Thus transfer of water against a chemical gradient might be secondary to active transport of a solute. Whether the water transport under these conditions can be considered to be active is a matter of definition. Only in very few cases of water transfer are all the necessary parameters known. This is, of course, especially true for water transfer across cellular membranes. Thus in many cases there is no agreement even about the magnitude of the activity of water in the cytoplasm (see, for instance, reference *64*). From an energetic viewpoint, the idea of a primary water transport is not very attractive. A simple stoichiometric relation between the number of water molecules transported and the number of, say, ATP molecules consumed in the transport process would make the transport very costly. If the transport mechanism is to work with a reasonable efficiency, so many water molecules would have to be transported per molecule of ATP that no simple reaction could form the link between the breakdown of ATP and the transport of water. In several cases the postulated active water transport may equally well be passive transfer caused by transport of some solute. Thus, for instance, the production of hypertonic urine has been considered as being very good evidence for the existence of active water transport. However, it has recently been claimed that it is unnecessary to assume participation of active water transport. The concentration may equally well be brought about by osmotically withdrawing the water from the collecting ducts. The necessary osmotic pressure difference would be maintained by the "countercurrent" arrangement of the loops of Henle, collecting ducts, and blood vessels, possibly in combination with active transport of sodium into the intercellular fluid by the loops of Henle (*65, 66*).

Recently a review on water absorption in the gut has been published (*24a*). It has been concluded that in rat intestine water absorption is secondary to solute transfer, i.e., there is no net water movement unless there is also a net solute transfer. Under normal conditions water leaves the lumen because of an active transport of sodium and chloride ions.

In the above treatment it is assumed that temperature gradients across membranes are nonexisting or of negligible importance. However,

TABLE I
Occurrence of Types of Active Transport

Substance transported	Tissue and/or organism	References[a]
Sodium	Kidney	*76, 77*
	Urinary bladder (toad)	*78*
	Red blood cell	*19, 21, 79*
	Muscle	*80, 73, 81*
	Nerve	*17, 82*
	Rumen	*83*
	Skin (frog)	*24, 5*
Potassium	Kidney	*30, 71*
	Red blood cell	*20, 79*
	Muscle	*84, 73*
	Nerve	*17, 73*
	Yeast	*31, 85*
Hydrogen	Kidney	*86, 71*
	Stomach	*9*
	Yeast	*32, 85*
Chloride	Stomach	*37, 87*
	Skin (frog, adrenaline stimulation)	*88*
	Root (wheat, also other anions)	*8, 89*
	Carrot disk	*10*
	Gill (eel)	*90*
Iodide	Thyroid	*41, 42*
Phosphate	Kidney	*91*
	Staphylococcus aureus	*44, 92*
Diodrast	Kidney (mutual competition)	*47*
p-Aminohippurate		*48*
Phenol red		*49*
Penicillins		*50*
Caronamide		*93*
Basic amino acids	Kidney (mutual competition)	*53*
Leucine *iso*-Leucine	Kidney (mutual competition)	*53*
Glycine Alanine Creatine	Kidney (mutual competition)	*52*
Glutamic acid Glutamine Glycine	Ehrlich mouse ascites carcinoma	*94*
Glutamic acid	*Staphylococcus aureus*	*46, 92*
Glucose	Kidney	*57, 72*
	Intestine	*56, 72*
Urea	Kidney (frog, not in mammals)	*95*
Oxygen	Swimbladder (cod)	*60*
Nitrogen Argon	Swimbladder (whitefish)	*61*

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[a] If more than one reference is given the first usually refers to an original paper and the other(s) to recent review(s).

the validity of this assumption has been questioned and a hypothesis for water transfer driven by a temperature gradient has been proposed (*67*).

Further examples of active transport may be found in several recent publications (*68–75*). Table I lists the occurrence of different types of active transport.

V. Energy Sources

As mentioned before, active transport requires energy supplied by metabolism, be it aerobic or anaerobic. Although many microorganisms are known to live under wholly anaerobic conditions, very little is known as to which substances they exchange with their surroundings. In aerobic organisms the evidence for active transport at the expense of anaerobic metabolism, i.e., glycolysis, is very scarce. Cation transport in fermenting yeast (*31*) and in red blood cells of some (very likely all) mammalian species (*19, 21*) are the only proven cases. As the nonnucleated red cells consume glucose but not oxygen to any extent, this is to be expected. Additional proof is given by the observation that even high concentrations of poisons interfering with oxidative metabolism [cyanide, 2,4-dinitrophenol (DNP), and malonate] are without effect on the active transport processes, whereas inhibitors of glycolysis (monoiodoacetate and fluoride) in low concentrations are greatly inhibitory (*96*). Likewise, in fermenting yeast, cyanide is without effect upon the accumulation of potassium ions, whereas monoiodoacetate and to a lesser degree fluoride not only inhibit the uptake of potassium but also cause a loss of this ion (*31*).

Recently it has been claimed that glycolysis could provide energy for active transport of sodium across the frog skin (*97*). The ratio between the number of equivalents transported under anaerobic conditions and the number of simultaneously produced moles of lactic acid varies between 4 and 9. As some of the energy in all likelihood is used for other purposes as well, the true ratio is probably higher. This implies that more than 4 ions are transported per mole of ATP provided by glycolysis. It is difficult to imagine a simple stoichiometric coupling between the breakdown of one mole of ATP and transport of more than 4 sodium ions. It is more probable that at least part of the energy has been derived from an energy reserve created under aerobic conditions.

In most other cases reported, there can be little doubt that the active transport processes derive their energy from aerobic metabolism, as indicated by the fact that poisons like cyanide and DNP are powerful inhibitors. An inhibitory effect of oxygen lack does not as such prove dependence upon oxidative metabolism, because oxygen may very well be essential for maintaining the membrane structure. Thus the small intestine is extremely sensitive to an inadeqate supply of oxygen, which even for short periods will lead to irreversible damage of the tissue.

It is interesting that nucleated red blood cells those of the chicken, for example (in contrast to nonnucleated red cells), can derive energy for active cation transport from aerobic metabolism (*98*). It was thought that nucleated cells were unable to derive energy from glycolysis. However, in duck red cells transport is faster under anaerobic conditions than when oxygen is present (*79*).

How energy is transferred from metabolism to the transport processes is not known. It has been claimed that phosphorylation is an essential step in the transport of some substances, e.g., glucose. The claim is based on the observation that phlorizin, a powerful inhibitor of phosphorylation, inhibits the uptake of glucose in kidney and small intestine. However, by inhibiting phosphorylation, phlorizin may act simply by cutting down the rate at which energy-rich phosphate compounds can be supplied. DNP, which is believed to prevent the production of energy-rich phosphate compounds by the oxidative metabolic cycle, also strongly inhibits many active transport processes. This fact lends support to the assumption that energy is transferred via energy-rich compounds like ATP.

More direct evidence for the linkage between energy-rich phosphate compounds and active transport processes has lately been published (*98a,b*). When isolated giant axons of the squid are poisoned with cyanide extrusion of sodium ions as measured with Na^{22} decreases. On injecting ATP or arginine phosphate the rate of Na^{22} appearance in the bathing solution is temporarily increased.

VI. Specific Inhibition and Stimulation

Quite often inhibiting and stimulating agents are used in an attempt to obtain further knowledge about the active transport mechanism. The inhibitory substances are almost all metabolic inhibitors. In more or less damaged preparations, such as tissue slices, stimulating effects are often obtained with substrates for metabolism. In more intact preparations metabolites generally are without effect, probably because they cannot permeate into the cells. Besides the ordinary cell poisons, the group of substances with an effect on relatively undamaged tissue comprises heavy metal ions, oxidizing agents, and hormones. It is notable that the steroid hormones, which in the intact organism cause salt retention, often have an inhibitory effect, if any, on salt transport in experimental preparations. Of the substances without a primary effect on metabolism, two may be worth-while mentioning, viz., ouabain (*g*-strophanthin, a cardiac glycoside) and the neurohypophyseal hormones.

Ouabain has long been known to affect the heart, causing a loss of potassium from the heart muscle cells. This release of potassium was thought to be responsible for the physiological effect. Recently ouabain and related drugs have been tested on striated muscle (*99*) and on red blood cells (*100, 101*). In both preparations only the drugs with a cardiac effect caused a decrease in the ability to extrude sodium and take up potassium. When administered to the inside bathing solution of the isolated frog skin, active sodium transport is inhibited. At the same time about 60% of the potassium in the skin is given off to the inside solution (*102*). In all cases mentioned, there are indications that the transport of sodium and potassium ions are interdependent. It seems very likely that the similar effect of ouabain on the different preparations reflects the similarities between the ion transport mechanisms.

While ouabain seems to interfere directly with the active transport mechanism, the neurohypophyseal hormones probably act by changing the membrane structure. When the hormones are added to the inside bathing solution of the isolated frog skin the rate of active sodium transport is increased (*24*). The oxytocic and the pressor principles both exert this effect (*103*). Since the permeability to passively penetrating substances is also increased (*104*), the apparent stimulation of sodium transport most likely consists in a decrease of the internal resistance to sodium and/or potassium ions.

VII. Quantitative Relation to Metabolism

Since the details of the energy transfer from metabolism to transport processes are not known we cannot do anything better than compare the work, W, performed during transport with the maximum amount of energy that can be released by metabolism. If the active transport depends on, say, aerobic metabolism, W can be compared with the maximum amount of work obtainable from the reduction of the oxygen consumed by the transporting tissue. The work that has to be done in active transport can conveniently be divided into three parts, namely, work to overcome (*a*) the concentration gradient, (*b*) the electrical potential gradient, and (*c*) the internal resistance of the membrane. (For the sake of simplicity we assume no net transfer of water to take place.) If the transport could be carried out in a reversible way the work required would be concerned with only (*a*) and (*b*). Under these conditions the work, in joules, performed in the transport of one mole of substance from side 1 to side 2 would be:

$$W = RT \ln c_2/c_1 + zF(\psi_2 - \psi_1)$$

The work may be calculated in calories by multiplying W by 0.239. If the internal resistance is too large to be disregarded, the expression will have an additional term. With certain assumptions a minimum value for this work may be obtained. Although the transport process as a whole is irreversible, the rate-limiting part may be either fully reversible or microscopically reversible, i.e., the process may be divided up into (infinitesimally) small steps, each individual step being reversible. Under these conditions an estimate of the minimum amount of work can be obtained in the following way. When active transport takes place there is a discrepancy between the flux ratio observed and the one calculated according to Eq. 2. However, one may add a term to account for the discrepancy between the predicted flux ratio and the one found, thus

$$RT \ln M_{12}/M_{21} = RT \ln a_1/a_2 + zF(\psi_1 - \psi_2) + zF(\pi_1 - \pi_2)$$

This equation may be said to define the *effective* active transport potential, $\pi_1 - \pi_2$. The last term in the above equation, $zF(\pi_1 - \pi_2)$, is the work performed per mole actively transported, thus

$$W = RT \ln a_2/a_1 + zF(\psi_2 - \psi_1) + RT \ln M_{12}/M_{21} \tag{4}$$

It is obvious that under conditions of net transfer an element of exchange diffusion will invalidate Eq. 4 by making the flux ratio smaller, thus resulting in too low a value for the last term in the equation.

The ratio 100(0.239 W)/(oxygen consumption) (caloric equivalent of oxygen) has been termed the energetic efficiency. The ratio is likely to give too small values for the true energetic efficiencies because all the oxygen is probably not used for the transport processes. If it is possible to increase the intensity of the transport processes with a stimulating agent, one can use the increments in W and oxygen consumption rather than the absolute values for calculating the energetic efficiency. However, one may be seriously in error if part of the metabolism under different conditions may be used for different purposes.

A membrane across which active transport of ions takes place may be considered as an electric battery. Like other batteries it will deliver the maximum current strength when the external resistance is zero. If epithelial membranes are dealt with, the maximum current strength may be measured by the short circuit technique (see Fig. 1). If ion transport is coupled directly to the reduction of oxygen, as assumed in the electron linked transport hypothesis, not more than 4 faradays would be generated per mole of oxygen reduced. The ratio 100(maximum number of coulombs generated by the membrane)/(coulombic equivalent of the oxygen consumed) is called the coulomb efficiency.

A. TRANSPORT INTO OR OUT OF CELLS

Before isotopes were obtainable the only way to study active transport processes across cellular membranes was the depletion-repletion approach. As the name indicates, the cells under study are depleted of some particular substance, whereupon the cells are allowed to regain the substance. This type of approach has been extensively used in experiments on muscle cells and red blood cells. In fact the active nature of both the sodium extrusion from muscle cells (*80, 105*) and the potassium uptake in red blood cells (*19, 21*) was demonstrated for the first time in this way. The depletion-repletion approach may still prove to be useful, as long as one is aware of the possibility that as the internal medium is changed the preparation may become something quite different from what it was at the start of the experiment.

As isotopes of the biologically important chemical elements became available, a new way was opened for the study of active transport in and out of cells. It is now possible to measure the flux in either direction in the steady state where no net changes take place. This is possible because the methods of analysis for isotopes far surpass chemical analyses in sensitivity. When a cell is in the steady state, the fluxes in and out of the cell are equal for all substances but those which are being consumed or produced inside the cell. However, this latter possibility will not be considered here. In the steady state, the active transport of a substance is counterbalanced by a diffusion in the other direction. As one-way diffusion is impossible, the flux due to active transport is smaller than the total flux. In most cases this difference is small, so the steady state flux may be used in calculating the minimum amount of work required to maintain the steady state condition for the substance in question (cf. Krogh, *106*). It is obvious that if any part of the transfer goes by way of exchange diffusion the calculation gives too high a value for the energy expenditure, since exchange diffusion is essentially "costless."

As an example of the steady state approach, we may consider the transport of cations across the red blood cell membrane (*79*). It will be recalled that for a substance which crosses a membrane by free diffusion we have: $M_{12}/M_{21} = \bar{a}_1/\bar{a}_2$, where $\bar{a}$ is the electrochemical activity. This may be written: $M_{\text{in}}/M_{\text{out}} = (c_{\text{out}}/c_{\text{in}})\exp(zFE/RT)$. In red blood cells, chloride ions are believed to be at thermodynamic equilibrium across the cell membrane. We then write $E = RT \ln[\text{Cl}]_{\text{out}}/[\text{Cl}]_{\text{in}}$ which for univalent cations gives $M_{\text{in}}/M_{\text{out}} = (c_{\text{out}}/c_{\text{in}})([\text{Cl}]_{\text{out}}/[\text{Cl}]_{\text{in}})$. Then, inserting the rate constants defined by $k_{\text{in}} = M_{\text{in}}/c_{\text{out}}$ and $k_{\text{out}} = M_{\text{out}}/c_{\text{in}}$ we obtain $k_{\text{in}}/k_{\text{out}} = [\text{Cl}]_{\text{out}}/[\text{Cl}]_{\text{in}}$. In human red cells the steady state fluxes are approximately 2 mmole per liter of red blood cells per hour for potassium, and 3 mmole per liter of red blood cells per hour for sodium. The internal and external concentrations of potassium are 140 mmole/kg. cell water and 4.5 mmole/liter, whereas the corresponding figures for sodium concentrations are 15 mmole/kg. cell water and 140 mmole/liter respectively. Thus for potassium $k_{in}/k_{out} = 0.636/0.020 = 32$ and for sodium $k_{in}/k_{out} = 0.031/0.286 = 0.11$. At the same time $[Cl]_{out}/[Cl]_{in} = 1.2$. If the chloride ions really are at or near thermodynamic equilibrium these deviations from the chloride concentration ratio indicate that the ions are actively transported or that the transfer is wholly by way of exchange diffusion. Since influx and outflux for both ion species may be varied the latter possibility can be excluded. If it is assumed that outflux of potassium takes place solely by diffusion, the rate constant for passive influx can be calculated to be 0.025, which is small compared with the total rate constant of 0.636. For sodium the passive outflux may be calculated to be 0.026 as compared with 0.286.

If it is assumed that exchange diffusion plays only a minor role, if any, the minimum energy required in the steady state can be calculated and compared with the concomitant glucose consumption. In the steady state, influx equals outflux. The flux ratio term therefore becomes zero. The potential term is calculated assuming thermodynamic equilibrium of chloride ions. If one uses the concentrations and fluxes given above, the work performed per liter red blood cells per hour becomes about 9 cal. At the same time about 1.5 mmole glucose are consumed, by which 75 cal. of free energy are made available. The over-all energetic efficiency thus becomes about 12%. So far no satisfactory measurements have been made of glucose consumption under varying cation transport intensities. This is not surprising, since only about one eighth of the total energy is being used for active transport.

B. Transcellular Transport

As examples of the efficiency of transcellular transport, two cases will be considered. The first example is the secretion of hydrochloric acid by the gastric mucosa. For a long time it was believed that the primary process was secretion of hydrogen ions. Not until recently has it been realized that chloride ions are actively transported, possibly by a forced exchange with bicarbonate ions (37). In a consideration of the energetic aspects of hydrochloric acid secretion, it is of little importance how the secretion actually is carried out. In principle the work can be calculated by using Eq. 4 for every substance secreted, including water. This is not feasible, however, when hydrogen ions are involved, since it is impossible to measure the two opposite fluxes of hydrogen ions. Fortunately the total resistance of the gastric mucosa is so small that leaving out the internal resistance term probably causes only a minor error in the calculation of W. Since electroneutrality has to be maintained, equal amounts of positive and negative charges must be transferred. Therefore, in the calculation of the minimum total work, the potential difference term can be disregarded. The expression for W_{total} is composed of terms of the form $RT \ln c_s/c_p$, one for each ionic or molecular species secreted, c_s and c_p denoting the concentrations in the secretion and in the plasma, respectively. Although the single terms do not yield the right values for the work required, W_{total} will have the proper value. Since the secretion is isosmotic with blood, the water term will become zero. In round numbers, the secreted acid is 0.15 N; hence W_{total} is about 10,000 cal. per mole hydrochloric acid. This value for the minimum amount of work may then be compared with the simultaneous oxygen consumption. When the amount of acid secreted is compared with the total amount of oxygen consumed, coulomb efficiencies less than 100% are practically always found (*107*). Since the acid secretion (oxyntic) cells constitute only about 10% of the total mucosal mass, this calculation does not seem to yield a fair estimate. If the increments in acid secretion and oxygen consumption during histamine stimulation are compared, coulomb efficiencies as high as 300% may be obtained, corresponding to 12 moles of HCl per mole of O_2 or 3 HCl per electron (*108*). One mole of oxygen furnishes well over 100,000 cal. when carbohydrate is the substrate. Assuming 100% energetic efficiency, about 12 moles of HCl can be formed. The limitations of this type of approach are clearly brought out if one considers stoichiometric relations. If it is assumed that the energy is provided via energy-rich phosphate compounds, say ATP, at most one mole of HCl could be transported per mole of ATP, the latest value for the energy of the terminal bond being about 7000 cal. per mole (*109*). If it is further assumed that 6 moles of ATP are formed per mole of oxygen reduced, the coulomb efficiency can at most be 150%, indicating that part of the metabolism can be switched from nontransport purposes to acid production, that part of the energy is supplied by glycolysis, or finally that ATP is not involved in the transport. Since DNP inhibits acid production, the most likely of the three possibilities seems to be that, on demand, some energy can be diverted from other purposes to acid production. However, it has been claimed that glycolysis also can provide energy for the transport processes (*110*).

As the second example of transcellular transport efficiency, we will consider the active transport of sodium ions across the isolated frog skin (*24*). When the skin is bathed on both sides with Ringer's solution, sodium ions will be transported from the outer to the inner bathing solution. Chloride ions are dragged passively through the skin due to electrostatic attraction, since the inner solution is positive relative to the outside. If the proper potential is applied from an external battery, the potential difference across the skin can be kept at zero. As the two bathing solutions are identical, the electrochemical activities for all substances are equal on the two sides. Hence only sodium ions will show a net transfer. Under normal conditions the net sodium transport is exactly equal to the current generated by the skin. If this "short circuit" current is compared with the total amount of oxygen consumed by the skin, coulomb efficiencies as high as 200% may be found (*11*). As glycolysis does not seem to play any role as a source of energy in these experiments, the electron-linked transport hypothesis evidently is unable to account for the high coulomb efficiencies. Since part of the oxygen must be used for other purposes than transport, the calculated efficiencies must be too small. Under the assumption that the "nontransport" fraction of the metabolism is a constant entity for each skin, changes in transport intensities and the concomitant changes in oxygen consumption have been used for calculating the coulomb efficiencies. Changes in the amount of sodium transported were brought about either by changing the supply of sodium for the transport mechanism or by means of neurohypophyseal hormones. In either case the coulomb efficiency was about 500%. This value for the coulomb efficiency may then be compared with the energetic efficiency. When the outside and inside solutions are identical and the potential difference across the skin is zero, the transport mechanism has to overcome only the internal resistance of the skin. The equation for the minimum amount of work performed during transport then becomes

$$W = 0.239\ RT\ \ln M_{\text{in}}/M_{\text{out}}$$

or

$$W = 1340\ \log\ M_{\text{in}}/M_{\text{out}}.$$

The flux ratio varies between 10 and 100 and hence W varies between 1340 and 2680 cal. per equivalent of sodium transported. Since the RQ of frog skin is close to one, one may take the caloric value of oxygen to be 100,000 cal. per mole. This means that about 5000 cal. are available for the transport of one equivalent of sodium, i.e., the energetic efficiency lies between roughly 30% and 50%.

If it is assumed that ATP provides the energy source, and that 6 moles of ATP are formed per mole of oxygen reduced, 20 sodium ions will be transported at the expense of the energy available from the terminal bonds of 6 moles of ATP. As mentioned above, the latest value for the energy of this bond is 7000 cal. per mole. This means that about 2000 cal. are available per equivalent of sodium. This value does not conflict with the values 1340 and 2680 cal. calculated above. The coulomb efficiency of 500% thus seems reasonable.

Even if the frog skin is allowed to perform uphill transport of sodium against a concentration gradient and an electric potential gradient, the coulomb efficiency remains the same, although the energetic efficiency is much increased.

The generally accepted assumption that the rate at which active transport proceeds is determined by the intensity of the metabolism has been shown not to hold true in some cases. On the contrary, the active transport rate can be an important factor in determining the metabolic rate. The so-called "anion respiration" (probably more correctly termed "salt respiration") in plants constitutes one example. As already mentioned, the oxygen consumption of carrot disks, for example, is increased when the disks are transferred from distilled water to salt solutions (*10*). Recent observations on the stimulating effect of ADP on metabolism of yeast and mitochondria suggest that if active transport is energized by breakdown of ATP (or the like) the higher metabolic rate following an increased transport rate is caused by an increase in the concentration of ADP (*111*). Some experiments on frog skin constitute perhaps an even clearer indication that the intensity of active transport may determine to some extent the magnitude of the metabolism (*112*). When the potential difference across the frog skin is artificially altered, both the intensity of the sodium transport and the oxygen consumption are changed. Thus the less positive the inside of the skin is relative to the outside, the more sodium is transported and the more oxygen is consumed. Since it is unlikely that a change in the potential difference would primarily affect the metabolism, evidently the active transport at least partly determines the metabolic activity. It has been observed that the change in oxygen consumption lags behind the change in sodium transport. This may indicate that the immediate energy is supplied from a pool of, say, ATP which is converted into ADP the level of which determines the metabolic activity.

VIII. Relation to Bioelectric Potentials

The existence of potential differences across the cellular membranes of muscle and nerve cells and across the frog skin was discovered in the early half of the last century (*113*). Since then many other biopotentials in plants as well as in animals have been reported. Although it was soon realized that the potential difference across a membrane influenced the movement and distribution of ions, almost a hundred years passed before it was realized that the biopotentials are themselves created by active transport of ions. This was first demonstrated in the frog skin, where under normal conditions sodium ions could be shown to be the only ions permeating in a nonpassive way (*24*). However, the magnitude of the spontaneous potential difference at any time is not determined exclusively by the action of the sodium pump. Any passive ion permeation constitutes a short-circuiting of the skin battery, thus resulting in a submaximal potential difference across the skin. Active sodium transport is undoubtedly responsible also for the potential difference across most other epithelial membranes (*78, 114*). In nerve cells and all kinds of muscle cells, the potential difference across the cellular membrane is in all probability also caused by active transport of sodium ions. (Sodium transport is taken to mean forced exchange of sodium ions against potassium ions, hydrogen ions, etc.). As already mentioned, the similar effects of ouabain on red blood cells and on muscle cells, as well as on frog skin, indicate that the same type of transport mechanism is at work in all these cases. Recently it has been observed that the actively maintained potential difference across the frog skin behaves as if it were composed of three diffusion potentials, namely, a sodium diffusion potential at the outward-facing border of the epithelial cell layer, a potassium diffusion potential at the inward-facing border, and a "leak" caused by the passive permeation of anions (*115*). By microelectrode studies of the skin potential profile, the existence of two distinct potential jumps has been demonstrated (*116*). The depths in the skin at which the two potential jumps occur are compatible with the assumption that the electric potentials are located at the two borders of the *stratum germinativum*. These and other observations have been interpreted to give the following picture of the active transport mechanism in the frog skin. The outer cell border is practically impermeable to cations other than sodium ions, which pass the membrane in a highly specific but strictly passive way. The direction of the electric potential gradient across this cell border is mainly determined by the relative sodium concentrations on the two sides of the cell membrane. At the inner cell border sodium ions leave the cell practically only by way of an active, forced exchange for other cations, presumably potassium ions. The inner cell border is highly permeable to potassium ions, which by diffusing back into the inside bathing solution, give rise to the second potential jump. The active sodium transport is thus the result of the cell's effort to maintain a low sodium and a high potassium concentration internally. It will be noted that the theory claims little more than an asymmetrical distribution of the cation transport mechanism assumed to be present in the cell membrane of most, if not all, animal cells.

In some other cases the chloride ion seems to be responsible for creation of biopotentials as, for example, in the gastric mucosa (*37*). In the frog skin active movement of chloride ions contributes to the potential difference during adrenalin stimulation, when an active transport of chloride ions outwards takes place, presumably through the skin mucous glands (*88*). Although it has not as yet been proved, it seems most likely that the secretion potential of the sublingual salivary gland is also caused by active secretion of chloride ions (*117*). It is interesting to note that the cells which are believed to transport chloride ions in animals histologically look like mucous gland cells. This is also true for the chloride-secreting cells in the gill of the eel (*118, 119*).

Biopotentials have been thought to arise from uneven distributions of ions on the two sides of a membrane impermeable to one or a few of the ion species present (Gibbs-Donnan potential). This explanation at present is not only unsatisfactory but also unnecessary.

It will be noted that in several cases active transport of ions is carried out, or seems to be carried out, as a forced exchange with ions of another species of the same charge, e.g., hydrogen ions and potassium ions in fermenting yeast, chloride and bicarbonate ions in frog gastric mucosa, and sodium and hydrogen ions in the distal tubule. Perhaps as more knowledge is gained about transfer processes, the active or "forced" exchange diffusion will prove to be generally involved in active transport of ions.

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CHAPTER 8

Balance of Water, Electrolytes, and Nonelectrolytes

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I. Introduction

A. The Concept of Balance: Limits of Present Treatment

We recognize an organism as a result of its chemical composition being different from that of its environment; that is, there exist chemical gradients across the limiting membranes of the organism. There is a tendency for the constituents of the organism to be dissipated under the influence of these gradients, the component molecules diffusing away from each other and eventually reaching a state of thermodynamic equilibrium where all differences of chemical potential have disappeared. A living system, however, not only retains its identity, and hence the prevailing electrochemical gradients, but does so in spite of a continual interchange of nearly all its constituent atoms with the atoms of the environment [see Schoenheimer (*1*), also van Bertalanffy (*2*) for a theoretical treatment]. The concept of balance implies that assimilation and excretion are so integrated as to preserve a constant steady-state level for the various components of the organism. High molecular weight components (the macromolecules, proteins, lipids, oligo- and polysaccharides) are, in general, retained within the organism by their inability to cross the limiting cell membranes and their steady state is a result of the balance between anabolism and catabolism. This direct consequence of metabolism will not be discussed here. It is fundamental to modern views, however, that the steady-state composition of most low molecular weight components is not due to their static retention behind impermeable barriers but rather to the active participation of the limiting membranes of the organism in the face of a definite permeability of these membranes. The maintenance of the balance of these diffusible components, water, electrolytes, and nonelectrolytes, will be the subject of the present chapter.

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B. MEMBRANE CONTROL

Diffusible components will move down the operative electrochemical gradients. The organism must do work to maintain such a component (say, Q) at either a higher or a lower electrochemical potential than the environment (unless the inwardly moving component can be removed by anabolism or catabolism, in which case the electrochemical gradient can be used to do work). The work done is the product of: the amount of the component which must be moved times the prevailing gradient. For a steady state the amount of Q which the organism must move against the gradient is clearly equal to the amount moving into the cell under the influence of the gradient. This amount is governed by the permeability of the limiting membrane to Q. The work done is least when the permeability is lowest. Hence it is of advantage for the cell to develop as low a permeability as possible for all components which have to be kept at a nonuniform electrochemical potential. Furthermore, the macromolecules of the organism require much free energy for their synthesis and it is a great advantage for the organism if they can be retained and hence not continually synthesized.

The limiting membranes can obviously not be made completely impermeable as this will prevent assimilation of essential nutrients. Present views (*3–5*) postulate that this selective permeability of cell membranes is in the first place determined by their structure as a bimolecular layer of lipid coated by protein. Substances diffuse through the lipid layer in proportion to their lipid/water partition coefficient. Thus the movement of polar molecules and of ions (both of which are often required to be maintained at a defined gradient) is reduced, but not the movement of nonpolar molecules (including oxygen and carbon dioxide).

The selectivity of the membrane is further increased by specific processes which increase the permeability of certain molecules and ions. Thus small polar molecules (water, urea, methanol, formamide) penetrate by a special mechanism, perhaps by diffusion through small aqueous channels (*6*). The rapid movement of anions across, for example, the erythrocyte membrane may also occur through such channels (*4*). Certain larger polar molecules, the pentoses and hexoses (*7, 8*) and glycerol (*7, 9*), penetrate by "facilitated diffusion" (*10*). The mechanism of this is obscure but appears to involve the formation of an intermediary complex with some component of the membrane. Such an enhanced permeability makes more difficult the maintenance of the balance of the component concerned, but the view is that these substances must be supplied to, or removed from, the cells at a sufficient rate in spite of a generalized low membrane permeability.

With the conditions for economic gradient control established, we must now consider the mechanism of maintenance of these gradients. This is the function of the "active transport" systems. Such systems, concerned in the transport of cell constituents against the prevailing electrochemical gradient, have been found for both electrolytes and nonelectrolytes and in numerous phyla. It will be shown in Section III that some active transport of ions is probably essential for maintenance of cellular integrity and thus is to be expected in all systems. The detailed (molecular) mechanism of active transport systems is discussed by Ussing (*11*). It is sufficient to emphasize here that active transport systems can be highly specific as would be expected on the view of their function outlined above. Thus the cation transport system in the human erythrocyte specifically pumps out sodium but not potassium (which is present in 7-fold greater concentration) or lithium (*12*). The specificity of an amino acid accumulating system in ascites tumor cells has been studied in great detail (*13*). In a manner reminiscent of enzyme specificity, slight modifications of the chemical structure of the penetrating amino acid have a marked effect on the activity of the concentrating system (cf. Table II). The active transport system concerned with the uptake of sugars from the intestine in mammals has been the subject of a number of studies which have demonstrated its specificity. Thus certain hexoses are rapidly absorbed but not the pentoses, which (being smaller) might have been expected to be absorbed the more rapidly (*14–16*) (see Section III). The system concerned in the uptake of sugars by yeast cells is also highly specific. Here, the specificity is similar to that of the yeast enzyme hexokinase which can phosphorylate those sugars that the cell takes up. Thus glucose, fructose, and mannose are readily absorbed but not galactose, sorbose, and arabinose (*17, 18*).

The active transport systems do thermodynamic work and the mechanism by which the metabolic free energy of the cell is channeled into their activity is being intensively investigated at the present time (*6, 11, 19*). That the free energy required does in fact come from the metabolic activity of the organism has been established in a number of cases, the technique being to inhibit metabolism in a manner which will have no direct (destructive) action on the active transport systems. As soon as the store of available energy is used up, active transport ceases. This has been demonstrated *inter alia* for frog skin (*20*), for mammalian red blood cells (*12*) and for crustacean nerve (*21*). Just how efficient is the use of metabolic free energy by the active transport system is not known, but the indications are that a good deal (up to 10%) of the metabolic energy of the cell is used for active transport (*22*).

The concept of the cell membrane consisting, in addition to the protein-coated bimolecular lipid layer, of a number of specific facilitated diffusion and active transport systems together with the energy-channeling components requires a far more complex model of the membrane than heretofore. Indeed, such a model has been put forward for the yeast cell (*23*), for the bacterial protoplast (*24*), and for the red blood cell (*25*). The most detailed model is that suggested by Rothstein for the yeast cell (*23*) which includes room for enzymes such as phosphatases and saccharases. In addition cation-binding sites and active-transport mechanisms are present. The classical permeability membrane is differentiated into three distinct barriers: to proteins, to divalent cations, and to univalent cations (see Fig. 1). Morphological investigation of the cell membrane has lagged behind this physiological analysis, even the most recent electron micrographs of, for example, the red cell membrane depicting a fairly undifferentiated simple plaque mosaic structure (*26, 27*). The electron-microscopic work has, however, confirmed earlier estimates of the thickness of cell membranes and confirmed the existence of the bimolecular layer (*28*). Further progress here awaits higher resolution studies and more important developments in increasing the specificity of electron-micrograph staining techniques. Studies using the electron-dense inhibitors of cell surface reactions (e.g. active transport systems) may well prove profitable.

In the following discussion, we shall lay emphasis on this membrane control, adopting for present purposes a model of the limiting membrane of the cell as being basically a macromolecular complex of, in general, low permeability. The permeability to certain substances is increased by specific membrane components, the facilitated diffusion systems, which enhance the rate of with-the-gradient movement of metabolites and waste products. In addition, the membrane contains active transport systems which by virtue of their linkage with free-energy-providing reactions allow the maintenance of electrochemical gradients for specific substances. In Section II we shall attempt to demonstrate that membrane control at those membranes of the organism which are in contact with the environment is so integrated as to preserve a balance and steady-state composition for particular components. In Section III we shall consider the role of membrane control in the maintenance of steady-state levels within different cells and tissues of various organisms. Section IV deals with problems of balance in plants.

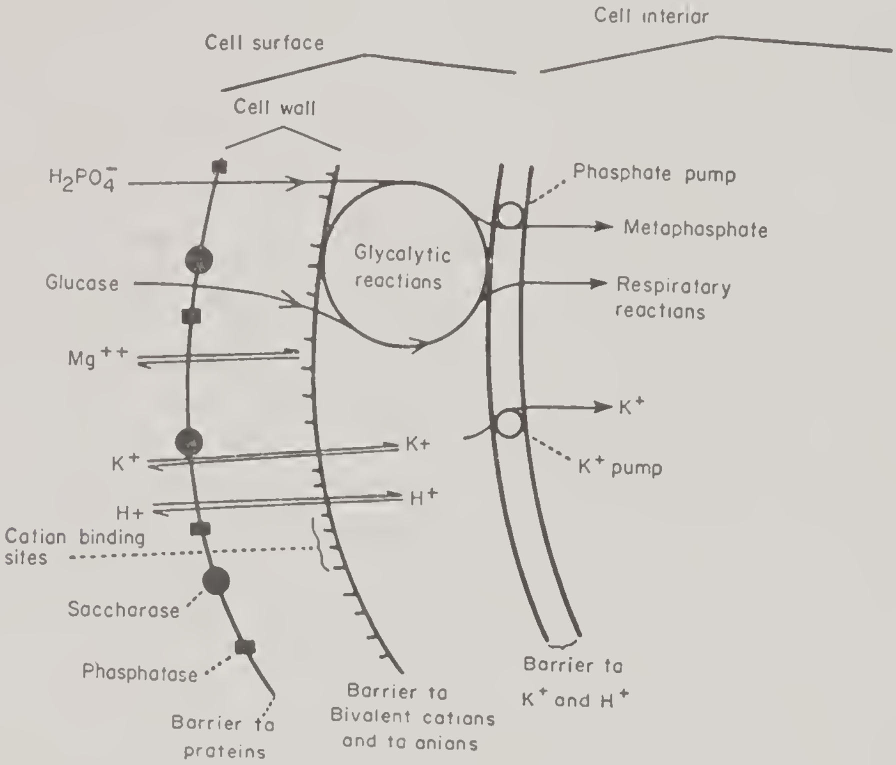

FIG. 1. Schematic representation of the cell surface of yeast. [After Rothstein (23)].

II. Balance in the Whole Organism

A. Historical Introduction

The concept of the constancy of the *milieu intérieur* is now so generally accepted that it is difficult to conceive of the situation which existed before Claude Bernard's statement: "La fixité du milieu intérieur est la condition de la vie libre" (*29*). Of this Haldane has said "No more pregnant sentence was ever framed by a physiologist." As Bernard stated: "Here we have an organism which has enclosed itself in a kind of hot-house. The perpetual changes of external conditions cannot reach it; it is not subject to them but is free and independent. . . . All the vital mechanisms, however varied they may be, have only one object, that of preserving constant the conditions of life in the internal environment" (*30*).

Since the time of Bernard all the accumulated evidence has supported his theory though at the same time broadening it to show how it is only by changes taking place in the internal environment that evolution can proceed. It has also become generally accepted that constancy does not mean an absence of physiological changes and that actually the internal environment fluctuates about a point of balance.

During the last thirty years the emphasis has shifted from the establishing of the fact of the constancy and has focused itself on the mechanism of the maintenance of the constancy of the internal environment. Out of this has grown the idea of active transport and the linking of balance to metabolic processes. Thus attention has been focused on the cell membrane as the important regulating structure.

This has had the desirable effect of showing that the maintenance of the constancy of the internal environment (which is achieved by specialized cells at the body surface and in the excretory organ) is not the only problem. As Ramsay has said (*31*): "We have been inclined to think of the cells of the body as being able to relax as it were, in a medium with which they are in equilibrium." It is now necessary to discard this idea and to try and visualize the cells as structures with individual balance problems which are solved by active transport processes between the cell protoplasm and the *milieu intérieur* (*31*). It is also important to understand how the balance of the organism as a whole is coordinated by nervous, muscular, hormonal, and behavioral activities.

B. General Concepts

In studying balance in the whole organism we shall consider:

(i) the amount of a particular substance in the organism compared with that in the external environment, i.e., the point of balance of that component;

(ii) the restoration of balance under physiological, pathological, and experimental variations;

(iii) the relation of the balance points to the environment, habit, and evolution of the organism;

(iv) where (and how) metabolic energy is involved in the maintenance of balance.

Accepting the hypothesis that the earliest organisms were marine we can consider the problems which such an organism must overcome. We have seen that it is probably necessary to maintain within the cell essential nondiffusible molecules, for example important anions. As a result the cell is faced with an osmotic inflow of water which can be compensated for (see Section III) by the development of an active extrusion of sodium (*32*). This will result in accumulation of, for example, potassium ions inside the cell and a deficit of small nonfixed anions in the cell compared with the external medium.

An attempt will be made to look for this postulated basic sodium transfer system in the lower marine animals, bearing in mind that an apparent concentration of, for example, potassium ions, may be a result of the active sodium extrusion. In other words, we should expect even the lowest animals to show powers of ionic regulation even in an isotonic marine environment. (Even if this view of the role of sodium extrusion is incorrect, some type of ion regulation will probably have to exist in order to overcome the "fixed anion" effect.)

Secondly it is necessary to consider the problems which arise as a marine animal becomes more complex. Differentiation of the cells responsible for entry and those controlling exit requires the setting up of coordinating mechanisms which may be nervous, hormonal, or behavioral. For example, a more complex organism will tend to have a large part of the surface in contact with the environment relatively impermeable and will maintain specialized regions for active processes (*31*).

The third problem is the effect of environmental change as a result of the progressive colonization of more dilute sea water and the adaptation to a fresh water environment. A simple animal of marine origin, the fluid content of which is isotonic with sea water, faces in a more dilute medium the osmotic entry of water across the semipermeable membrane. One way of coping with this is to develop a less permeable membrane. This however is only possible up to a certain extent since the animal has to be able to take up necessary substances from the medium and to get rid of unwanted metabolic products. A second method is the extrusion of water which has entered osmotically either actively as such or alternatively by the formation of some sort of ultrafiltrate from which salts are actively reabsorbed. As the animal becomes more complex this will tend to result in the development of a special organ for osmoregulation. One factor which helps to reduce water entry is the loss of ions to the environment so that in diluted sea water the ion concentration of the animal is reduced (*33*).

What happens to the "fixed anion" effect in a more dilute environment is a matter for speculation. Initially there will certainly be a tendency to lose ions to the environment as the active sodium transport process will pump them out and also diffusion outwards with the gradient will occur on dilution of the external medium. Though the animal can afford to lose some ions there must come a point when it has to conserve them. One method of doing this is to take up ions actively from a lower external concentration. If, however, the primitive extrusion, for example, for sodium, is still functioning, any uptake would be competing with this and the animal would be highly inefficient. It thus seems reasonable to suggest that the extrusion mechanism is either lost or reduced in those cells actively taking up ions; alternatively, it is interesting to speculate as to whether the mechanisms developed primitively for extrusion could in fact be reversed and used for accumulation (see later for a discussion of chloride secreting cells in *Fundulus*). Simple reversal would, of course, have to be combined with new active processes, for example for K^+ ions, in order to maintain the correct ion ratios on which metabolic processes had come to depend.

Fourthly it is necessary to look at changes taking place when a freshwater animal reinvades the ocean. Such an animal would have tended to maintain an ionic concentration lower than that found in the sea and thus the animal has either to increase the tonicity of its body fluid and cells, or to find a way of maintaining its ion concentration hypotonic to the medium. A further problem will arise because the freshwater animal will have organs for osmoregulation which will be a disadvantage in a hypertonic environment. Interesting cases will be found in those animals which are able to live in two differing environments and naturally adapt to each.

The adaptation of a freshwater animal to land is a fifth important problem. In the terrestrial habitat the conservation of water becomes of primary importance and changes must take place to cope with this. In the first place the development of some protection against water loss by evaporation would be of great value. Secondly, the osmoregulatory mechanism must be modified.

The secondary aquatic habitat of some primitive land forms again presents its own problems. They may be those of an animal adapted for water conservation finding itself in a medium where it is necessary to get rid of a large volume of water.

Regulating mechanisms require energy. In a simple organism, most of the surface of the animal will be in contact with the environment and will be involved in using energy for active transport. In a more complex organism there are developed certain specialized cells (separating the body fluid from the external medium) which use energy for transport purposes and relieve the strain on the rest of the organism by buffering it against an unfavorable environment. In addition to this direct effect, the complex animal requires a mechanism for coordination of the balance of the organism as a whole and this is mediated by nervous, muscular, hormonal, and behavioral activity which again requires the expenditure of energy, though in a more indirect way.

C. Review of the Major Groups

In considering balance, we require data on the composition of the protoplasm in the cell compared with the medium surrounding it, whether this be body fluid or the external environment. The composition of the body fluid must also be compared with the external environment. It will be seen that, although the latter type of data is widely available, information on the actual chemical composition of cells is far from comprehensive, and further work on this aspect is obviously desirable. The difficulties inherent in such an investigation are discussed in Section III.

Thus ionic regulation is not only the maintenance in a body fluid of ion concentrations differing from those in the external medium (*34*), but is essentially the maintenance of such concentrations inside the cell compared with the immediate environment (*31*).

Much of the following account is taken from the excellent reviews of Prosser (*35*) and Beadle (*36*):

1. *Coelenterates*

The coelenterates can perhaps be regarded as the group containing the simplest primitive marine animals without any localized organs for excretion or absorption. Some mechanism must, however, exist for overcoming the basic problem of the "fixed anion" effect.

Analyses of the juice obtained by liquefaction of the body of *Aurelia aurita* and *Cyanea,* large marine medusoids, by Macallum (*37*) showed higher potassium (144–256%) and much lower sulfate (42–88%) than in the surrounding sea water. Robertson (*34*) repeated the analysis on the mesoglea (the fluid between ectoderm and endoderm) of *Aurelia* and showed low sulfate but no high potassium. It thus was suggested that the previous high K^+ was due to the inclusion of cell K^+ in the fluid. Thus there is indirect evidence of accumulation of K^+ by the cells bounding the mesoglea. Whether this is an active accumulation or the passive result of active sodium extrusion cannot at present be decided, and it is obvious that a great deal remains to be done on the ectoderm and endoderm cells of the marine medusoids. The contractile elements of these animals occur in the cells bounding the mesoglea, and it is possible that the contractile mechanism depends on a differential distribution of ions as in higher animals (*38*).

In the freshwater coelenterates, e.g., *Hydra,* there are two problems. Firstly, how to keep the ions inside the animal, and secondly, how to get rid of water entering osmotically. Lilly (*39*) showed that *Pelmatohydra* is permeable to sodium, potassium, and bromide and that these ions are actively absorbed from very low concentrations. She also suggested that the internal osmotic pressure of *Hydra* is equivalent to 0.04-0.05 *M* sucrose. *Hydra* is highly permeable to water and the endoderm cells contain vacuoles. However, the vacuoles are not known to contract, and in distilled water they enlarge and the animal disintegrates. Some mechanism (at present unknown) is therefore necessary to control water entering osmotically.

2. *Protozoa*

This latter problem of osmoregulation in fresh water has been widely studied in the Protozoa (*38*). It is now generally accepted that the function of the contractile vacuole (found universally in fresh water but only rarely in marine and parasitic forms) is to remove water entering by osmosis. For example, in *Pelomyxa* the osmotic pressure is equivalent to 0.09 *M* nonelectrolyte compared with 0.007 *M* in the culture medium (*40*). It also seems to be generally agreed that the contractile vacuole fills at a constant rate or even with acceleration and a simple osmotic theory of filling is therefore not accepted. The mechanism of emptying is also unsolved. It has been suggested (*41*) that a contractile protein in the vacuolar membrane may be involved. Secretion then may be according to the theory of Goldacre (*19*) who suggested that an unfolded protein could absorb certain substances, e.g., water, which would be released when the protein folded up reversibly. It is suggested that the necessary energy is derived from the oxidative metabolism of the cell.

3. *Annelids*

The marine polychaetes are good examples of animals which have become quite complex while still inhabiting their primitive marine environment. The body surface in contact with the sea appears to be still relatively unspecialized as far as permeability is concerned. However, nephridia are present and, as balance in all animals from the platyhelminthes upwards is tied up with nephridial or renal tubular function, the general problem of so-called excretory organs will be discussed here.

Goodrich (*41a*) considers that nephridia appeared early in the evolution of the Triploblastica and suggests that they must have been present in the ancestor of all the known phyla even before the development of a coelom. The flame cell of platyhelminthes probably represents the primitive type which Goodrich terms a protonephridium. He suggests that primitively such structures were concerned with the elimination of waste products and the regulation of the water content of the tissues. In a marine animal, however, neither of these functions would seem likely as the nitrogenous waste could diffuse from the body surface and water regulation is not necessary in an animal isotonic to the medium. Prosser (*35*) suggests that the nephridium is primitively an organ for disposing of unwanted ions, particularly Mg^{2+} and SO_4^{2-}. Perhaps a more likely explanation is that the first stage in nephridial development involved a tucking in or folding of a small region of the body surface and the cells lining this tube maintained their primitive function of Mg^{2+} and SO_4^{2-} secretion. Such a structure would obviously have great potentialities during evolution. The development of a bulb at the closed end which was in contact with the body fluid would allow ultrafiltration to occur and the cells lining the tubule could become adapted for selective absorption from the filtrate, thus permitting both nitrogen excretion and water elimination. The secretion of, for example, Mg^{2+} and SO_4^{2-} by the tubule cells could still be maintained and even enhanced.

If this type of view is correct it is necessary to view Mg^{2+} and SO_4^{2-} excretion as a primitive necessity. Why this should be so is not clear. It is not sufficient to suggest that these ions are poisons and therefore must be eliminated, as their poisonous properties are more likely to be a secondary consequence of the primary elimination. It may be that the initial elimination was an accidental consequence of some other process, for example, active sodium extrusion. It is known that Mg^{2+} and SO_4^{2-} have an effect on sodium transfer in red cells [see Davson and Danielli (*3*)] and Mg^{2+} also affects sodium transfer in the excretory tubule of the crab *Pachygrapsus crassipes* (*42*). (Though this effect could, of course, be secondary.) A second possibility is that the elimination of Mg^{2+} and SO_4^{2-} is in some way related to the solubility properties of the ions, for example, Ca^{2+} and PO_4^{3-} are both important ions physiologically and it may be important to prevent precipitation of calcium sulfate and magnesium phosphate. It is interesting that in the stick insect the serum and hemolymph are supersaturated with magnesium phosphate (*43*).

Goodrich (*41a*) suggests that in arthropods, mollusks, and vertebrates the nephridial function has been taken over by coelomoducts which are functionally analogous but not homologous.

Of the annelids, the purely marine form *Amphitrite brunnea* was shown (*44*) to have a higher concentration of potassium in the body fluid than that in sea water ($K^+_i/K^+_o = 1.43$) and this suggests some power of ionic regulation. But here too there is no data on cell concentrations which would be of great interest.

A great deal of work has been done on *Nereis*. Most species are marine, for example, *Nereis pelagica* and *N. cultrifera*, but *N. diversicolor* is also found in brackish water in estuaries. Unfortunately, as pointed out by Beadle (*36*), analyses of the cells and body fluids of these polychaetes are not available and without such data the question of active ion regulation in marine and estuarine forms cannot be discussed properly. However, Fretter (*45*) showed a rapid exchange of Na^{24} through the body wall from the body fluid to the sea water in *N. cultrifera* and less rapidly in the estuarine form *N. diversicolor*. But at present there appears to be no good evidence for the expected active ionic regulation in the marine or brackish water forms. *N. diversicolor* does, however, maintain a hypertonic body fluid in diluted sea water though its concentration is less than that found in normal sea water. Thus one suspects that there will be found in *N. diversicolor* the beginnings of hypertonic ion regulation, i.e., the ability to absorb ions from the medium against the gradient (see Section II,C,4). Beadle (*36*) suggests that this may be the accidental by-product of the more primitive ion regulation which, he thinks, will be shown to exist in the purely marine nereids. Again, on the "fixed anion" hypothesis such a primitive regulation is essential.

The maintenance of a hypertonic body fluid requires the elimination of water which enters osmotically. It is suggested that in *N. diversicolor* this may take place by the production of a hypotonic urine (*35*). There is morphological evidence for this as the nephridial canal of *N. diversicolor* is long and coiled and could provide a large surface area for reabsorption, whereas the nephridium of *N. cultrifera* is a simple sac.

In the presence of cyanide or lack of oxygen, *N. diversicolor* loses its power of osmoregulation and becomes like *N. cultrifera* thus indicating the active nature of the regulating process and its dependence on metabolic energy. Thus, it would appear that in fresh water the nephridium becomes modified for salt reabsorption and elimination of water.

Another factor influencing the survival of *N. diversicolor* in diluted sea water may be the ability of its tissues to continue functioning when the ionic concentration of the medium is lowered. This was shown by

Wells and Ledingham (*46*) for isolated muscle which still functioned in the dilute sea water of its normal estuarine environment.

A similar situation appears to exist in another estuarine polychaete, *Arenicola marina* (the lug worm). The animal has no osmotic control but the tissues still function in the diluted medium. However there may again be primitive ionic regulation as Robertson (*34*) showed that in dilute sea water the concentration of potassium in the body fluid was increased and that of sulfate was reduced.

Thus in the polychaetes adaptation to brackish water may originally depend on the ability of the tissues to withstand dilution followed by (or as a result of) adaptation of the primitive ion regulation mechanism to the maintenance of a hypertonic body fluid and eventually, adaptation of the nephridium for the production of a hypotonic urine. Potts (*33*) points out that the production of a hypotonic urine reduces the osmotic work necessary to preserve balance.

Some work has been done on the oligochaetes, in particular on two earthworms which live in contact with soil water and will survive in aerated fresh water. Using *Pheretima posthuma* Bahl (*47*) showed that the urine was hypotonic to blood and body fluid. *Lumbricus terrestris* is an interesting case which has been investigated by Ramsay (*48*). In fresh water the body fluid is equivalent to 0.53% NaCl, the blood is slightly less and the urine is very hypotonic, equivalent to 0.1% NaCl. Ramsay found evidence for active salt reabsorption in the wide section of the nephridium. Thus the nephridium is functioning as an organ for the removal of water and Ramsay points out the analogy with the primitive vertebrate kidney. A similar mechanism for salt reabsorption is located in the antennal gland of the crayfish, *Potomobius astacus,* again a freshwater animal.

The production of a hypotonic urine due to active salt reabsorption in the excretory organ appears to be a typical method of coping with the problem of salt conservation and water elimination in a freshwater environment and a mechanism for this has been developed independently in different groups of animals (*33*).

4. *Crustacea*

A great deal of work has been done on ion regulation in the Crustacea (*49*). These animals are marine in origin and can live in a wide range of salinities from pure marine to land. Prosser (*35*) arranges them in a series of seven groups according to osmotic capacity.

a. Animals limited to sea water which show no regulating capacity when put into dilute sea water, for example, *Maja,* the spider crab. It survives only a few hours in sea water diluted more than 20% (*50*).

The concentration of the blood follows the osmolarity of the medium. It is suggested that the urine produced is part of the usual process of ion regulation, i.e., elimination of unwanted ions.

b. Crabs with a limited ability for regulation which can therefore live in brackish water, e.g., *Cancer* and *Eriphia* (*50, 51*). Though the blood of *Cancer* is isotonic with sea water it contains more K^+, Na^+, and Ca^{2+} and less Mg^{2+}, Cl^-, and SO_4^{2-} than sea water. The urine is isotonic with the blood but contains more Mg^{2+} and SO_4^{2-} and less Na^+, K^+ and Ca^{2+} than the blood. This ionic balance is maintained by at least two active regulating processes, one concerned with uptake and one with output. In diluted sea water the blood is hypertonic though the urine remains isotonic with the blood. Thus the adaptation to brackish water must be a result of active uptake of ions, e.g., Na^+, K^+, and Cl^-, which takes place mainly across the gills; this adaptation also is helped by the animal having a lower permeability than *Maja* to water and salts (*51, 52*). The excretory organ plays no part in the adaptation. It is not known what regulating mechanism fails as the medium becomes more dilute.

c. Crabs which regulate better than those in category *b* in dilute sea water and live on the shore where freshwater streams empty, e.g., *Carcinus* (*53*) and *Hemigrapsus.* The body fluid is hypertonic in dilute sea water but the urine is isotonic with the blood and therefore the excretory organ plays no part in the osmoregulation but presumably still plays its primitive role, for example as an organ of excretion of Mg^{2+} and SO_4^{2-} and nitrogen (*54*). *Carcinus* cannot live indefinitely in sea water diluted much more than 40%.

The hypertonic body fluid is the result of two effects: (i) the permeability to water and salts is very low (*52*); (ii) the powers of ionic regulation are high.

In a dilute medium *Carcinus* uses more oxygen, presumably to maintain the active transport.

d. Highly regulating crabs. They live in very dilute brackish water and may go onto land. They can also regulate in a medium more concentrated than sea water, e.g., *Uca, Heloecius, Pachygraspus,* and *Palaemonetes.* They do not, however, live in fresh water.

The blood is hypertonic to the medium in dilute sea water and hypotonic in concentrated media (*55*). In the land crabs the chitinous shells afford good protection against water loss by evaporation.

In *Palaemonetes varians* Lofts (*56*) showed that a population living in water of highly salinity had a minimum respiratory rate in a medium of 2.6% NaCl in which the animal's blood was isotonic. Thus at this salinity least energy was being expended. A second population from water of much lower salinity had a minimum respiration rate in a medium of 0.6% NaCl. Thus in these two populations there is evidence of physiological adaptation to the environment.

In a recent paper Green and associates (*56a*), working on two species of *Uca* (fiddler crab) mainly in normal and more concentrated sea water, showed that the chief sites of the entrance of water and electrolytes are the stomach and gills. The chief regulating sites are the antennary glands and gills and possibly the stomach and mid-gut gland.

Gross (*54a*) working on a series of decapod Crustacea showed that *Emerita, Callianassa, Upogebia, Cancer antennarius, C. gracilis,* and *Pugettia* cannot regulate osmotically while *Pachygrapsus, Birgus, Hemigrapsus,* and *Uca* can do so. The osmotically regulating group have less permeable exoskeletons than the nonregulators by a factor of 3. Gross also showed that the respiration rate was greater when the crabs were subjected to greater osmotic stress. However, he suggests that the increase in metabolism is not a direct reflection of increased osmotic work but of muscular activity and other activity resulting from the changed external environment.

Gross (*54b,c*) suggested that in the shore crab, *Pachygrapsus*, K^+, Ca^{2+}, and Mg^{2+} are contained in extravascular pools in greater concentration than they are in the blood and that these pools play a part in the exchange between the animal and the medium. They would help to maintain osmotic and ionic constancy of the blood, e.g., in estuarine forms which could make up an osmotic deficit from the salt pools at low tide and low salinities and replenish the pools when the salinity was elevated on the incoming tide. But when desiccated, K^+, Ca^{2+}, and Mg^{2+} might shift from the pools to the blood and this physiological failure might limit their terrestrial life.

e. Crabs able to regulate so well either in concentrated or dilute media that they can live indefinitely either in the sea or in fresh water. For example, *Eriocheir* breeds in the sea and then migrates up European rivers where it grows to maturity. The mechanisms of adaptation are similar to those found in *Carcinus*. The permeability of the gills is very low, and as the urine is not hypotonic even in fresh water the hypertonicity of the blood in fresh water must be maintained by active uptake of ions. Krogh (*57*) showed rapid uptake of salt via the gills against a concentration gradient when an animal was depleted of chloride. Loss of ammonia appears to be extrarenal, for example, via the gills, since closing off of the kidneys does not affect it. Hence the kidneys are presumably acting as organs of excretion of unwanted ions as the salt composition of the urine differs from that of the blood; active transport must occur somewhere in the kidney.

f. Freshwater Crustacea. The purely freshwater crab *Potamon* maintains a high osmotic blood pressure (350 mM) (*58*), but the urine is still isotonic. The four anterior gills actively take up chloride (*58*).

In the crayfishes, however, a new phenomenon has appeared, i.e., the production of a hypotonic urine (*59*). The reabsorption of salts or secretion of water takes place in the long excretory canal of the antennal gland and thus hypertonicity of the blood medium is maintained. (In sea water isotonic with the blood the output of urine falls to zero. In this animal the concentration of all ions in the urine is less than that in the blood and therefore the kidney is not functioning as an ion excretory organ. It could be argued that this is a secondary condition following the adaptation of the antennal gland to water excretion.) In addition, the same factors operate as were found in the adapted crabs, i.e., low permeability to salts and active uptake of salts via the gills.

The oxygen consumption of the crayfish is 30–40% higher in fresh water than in sea water. It is suggested (*35*) that part of the extra energy is used in the active processes preserving the ionic balance of the animal.

In a recent paper Shaw (*59a*) suggests that *Potamon* shows a different type of adaptation to fresh water than that shown by the crayfish. It would appear ill-adapted as (*a*) the blood concentration is relatively high, (*b*) the body is relatively permeable to salts, and (*c*) it produces urine isotonic to the blood. But *Potamon* is 12 times less permeable than *Carcinus* and uses only 2.3% of its metabolic energy for osmoregulation. Because of this reduced permeability, salt and water loss through the urine is small despite the isotonicity of the urine. Shaw comments on the theory of Potts (*33*) that adaptation to fresh water is by (*a*) reducing the blood concentration and (*b*) producing hypotonic urine, neither of which tendency is shown by *Potamon.* This theory is, however, based on the assumption that the animal is semipermeable, a situation which may well be true in *Astacus* but is not found in *Potamon.* Shaw therefore extends earlier theories on stages in the adaptation to fresh water: (*a*) the maintenance of high blood concentration in dilute solution by the reduction in the permeability of the body surface together with the development of a salt uptake mechanism and (*b*) a further reduction in permeability. If the latter involves an all-round reduction in permeability to both water and salts, as in *Potamon,* then there is no need for decreases in either blood or urine concentration. If, however, the reduction in permeability is restricted mainly to salts, as in *Astacus,* then it is of value to reduce the blood concentration further and to produce a dilute urine.

g. Animals living in concentrated sea water, for example, *Artemia*, the brine shrimp from Great Salt Lake. The blood is hypotonic to the medium and the permeability is low but there must be active salt excretion, possibly via the gills which are enlarged in concentrated saline (*60*).

Recent papers by Croghan (*60a,b,c,d*) show that in *Artemia salina* the first ten pairs of branchiae (but not the eleventh) are the site of active NaCl excretion in media hypertonic to the blood and probably the site of active uptake in media hypotonic to the blood. *Artemia* continuously swallows the medium and Croghan considers that the gut actively takes up water and sodium chloride. Thus *Artemia* is adapted to the hypertonic medium in a similar way to the marine teleosts.

A series of prawns (subfamily Palaemoninae) living in sea water, brackish water, and fresh water shows similar tendencies to those mentioned in the crabs (*61*).

Rao has recently shown (*61a*) that in two groups of *Metapenaeus monoceros* (prawn) from marine and brackish water environments there is an increase in oxygen consumption with a decrease in salinity of the medium below that of the habitat.

5. *Mollusks*

The mollusks are primarily marine, and the blood is usually isotonic with sea water. Analyses by Robertson (*34, 62*) indicate considerable powers of ionic regulation when the blood is compared with the external medium. In the series lamellibranchs, gastropods, cephalopods, there is an increasing ability to regulate most ions, for example, potassium is twice as concentrated in the blood of the squid *Loligo*, as in sea water. Robertson suggests that the increased K^+ in the blood of cephalopods is correlated with the increased activity of the group, as activity depends on the maintenance of high K^+ in the nerves and muscles. Robertson showed that the excretory organs are also regulatory.

The freshwater bivalve, *Anodonta*, is an interesting case. It has the lowest recorded blood osmotic pressure (equivalent to 3% sea water) and produces a hypotonic urine by ultrafiltration from the heart into the pericardial fluid and active reabsorption takes place from the excretory tube through which the pericardium opens into the mantle cavity (*63*). The freshwater lamellibranch, *Hyridella australis* also has a low blood osmotic pressure and hypotonic urine.

Thus in the mollusks a similar pattern emerges as in other groups, i.e., the independent development of hypotonic urine production helping to maintain the low but still hypertonic (compared to the medium) blood concentration.

6. *Insects*

Certain groups of arthropods have become terrestrial and solved the problems of balance of water and electrolytes in a nonaquatic environment, for example, insects, spiders, scorpions, onychophorans, and isopods. Of these the insects have been studied most.

The main problem is the preservation of water, and the first requirement is the prevention of water loss by evaporation. Hence the very great importance of the chitinous cuticle which is nearly water impermeable (*64*). The greatest water loss is via the spiracles. Dilute urine is probably formed in the Malpighian tubules and water reabsorbed actively in the rectal glands. Thus the nitrogenous waste and also the feces are eliminated in a semisolid form.

In many land insects water is replaced by drinking; some, for example, clothes moths, wax moth larvae and meal worms, get most of their water by metabolic oxidation. In the latter case, water balance is maintained not by using metabolic energy but as a by-product of metabolism. In the case of the replacement of water by drinking, we have the coordination of the whole animal in a rather more complex way than in more primitive animals.

Adaptive mechanisms for maintenance of water balance may also be behavioral. For example, certain insects select a region of optimal humidity, e.g., *Tenebrio molitor* (meal worm beetle), *Culex fatigans* (the mosquito), *Blatta orientalis* (the cockroach), and *Pediculus humanus* (the louse (*65, 66*). Specific humidity receptors are probably located on the head and antennae.

It is now generally accepted that aquatic insects have evolved from terrestrial ones (*36*). The problem is therefore the modification of the land insect type for a successful aquatic life. The return to water has undoubtedly been facilitated by the presence of the cuticle with its low permeability to water and salts. On the other hand, as conservation of water is no longer so important, the cuticular permeability has increased (*67*). In addition there may be a special part of the body surface which is permeable to water and salts and permits limited exchange between hemolymph and the external medium. For example, the anal papillae of *Aedes*, *Culex*, and *Chironomus* (*68*). There is also ion regulation in the excretory organs and the fluid from the Malpighian tubules passes into the rectum where reabsorption may take place, for example, of K^+, Na^+, and Cl^- (*67, 69*). This rectal regulation can maintain remarkable constancy of Na^+ and K^+ in the blood of *Aedes aegypti* in the face of wide variation of these ions in the medium. Ramsay is of the opinion that the formation of urine in the Malpighian tubule is not by ultrafiltration but by selective excretion.

In a recent paper Ramsay (*69a*) has investigated the excretion of amino acids, sugars, and urea by the Malpighian tubules of the stick insect, *Dixippus morosus.* He shows that they enter the urine by passive diffusion and are absorbed by the rectum. He suggests that the tubule is primarily a means whereby all soluble substances of low molecular weight are removed from the hemolymph and that in this respect it has analogies with the glomerulus as well as with the tubule of the vertebrate nephron.

There is little information available on the source of the energy required for the active transport of ions in invertebrates. Though there is evidence for increased respiration rate under conditions of increased ion regulation, this in itself is insufficient data. Further work would be desirable on the effect of, for example, metabolic inhibitors on specific transfer processes.

7. *Protochordates*

a. Amphioxus. This marine animal possesses flame cells homologous to the protonephridia of the invertebrates. There is no development of coelomoducts for balance purposes as in other chordates.

b. Hemichordates. No nephridia have been reported, but coelomoducts not functioning as genital ducts are present.

c. Tunicates. These entirely marine animals possess neither nephridia nor excretory coelomoducts. The blood is isotonic with sea water but there is some ion regulation, for example, a very reduced blood SO_4^{2-} and HCO_3^- in *Phallusia* (*62*). It would be reasonable to suggest that the tunicates have lost the excretory organs presumably present in their ancestors, possibly because of a reduced activity. The ion regulation is thus carried out by other cells.

8. *Vertebrates*

The protonephridia probably present in the chordate ancestor have been lost in the vertebrates and their function taken over by coelomoducts which have become renal tubules (*41a*).

a. Fishes. There is at present considerable controversy about the origin of vertebrates. It is suggested (*70, 71*) that the early fishes arose in fresh water and that there have been periodic migrations to the ocean followed by reinvasion of fresh water. On the other hand, Gross (*72*) is in favor of a marine origin followed by a sharp shift to fresh water in the early Devonian and a reinvasion of the sea afterwards. This latter view is also supported by Denison (*73*) and Robertson (*74*).

As it is obviously difficult to obtain conclusive evidence from paleontological data the above authorities have relied on the interpretation of the significance of kidney structure and function in the fishes.

Romer accepts the view of Smith that the primitive vertebrate kidney was the organ of a freshwater animal whose functions were the conservation of ions and the elimination of water which has entered osmotically. Thus the marine teleosts and elasmobranchs are secondarily marine, and have to cope with the problem of water conservation in the face of a kidney adapted to water elimination. This would appear to be the only reasonable explanation of the two differing mechanisms found in the two groups of marine fishes (see later).

This line of argument, however, only confirms the theory that the immediate ancestor of the marine teleosts and elasmobranchs originated in fresh water, and does not rule out the possibility of the series marine → fresh water → marine put forward by Gross. In fact, analogy with the nephridia of invertebrates would favor this series. If the primitive nephridium was marine in origin, it is not unreasonable to suggest that the primitive vertebrate kidney was marine, and concerned, not with water elimination, but with ion regulation. As a marine invertebrate invades fresh water, we have seen the change in nephridial structure and function as the organ is used for conservation of ions and water elimination. Similar changes might be expected in a primitive vertebrate moving from marine to fresh water, and the result would be the kidney of the freshwater fish.

While such speculation cannot be used as evidence for one theory or another, it does show that kidney structure should not be used as evidence in favor of a freshwater origin of the fishes.

In considering balance in the fish, the relative impermeability of the skin, because of the scales and mucous, is important, but the gills and oral membranes are very permeable to water (*75*). In fresh water this water load is dealt with by the kidneys. The filtrate from the glomeruli passes along the tubules and most of the salts are actively reabsorbed along its length leaving a hypotonic urine, for example, in the gar pike in fresh water where the freezing point depression $\Delta = 0.03°$ the blood concentration is $\Delta = 0.57°$ and the urine $\Delta = 0.08°$ (*71*). However, some of the salts are still lost and must be replaced. This is achieved partly from the food and also by active absorption of ions from the medium via the gills. Krogh (*75*) showed salt loss by keeping freshwater fish in running distilled water. In dilute salt solution they then absorbed chloride against a gradient, for example, *Ameiurus* (a catfish) took up ions from 1 m*M* NaCl or NaBr.

For such a fish in the ocean it is a disadvantage to have a water-eliminating kidney since the main problem is the conservation of water and the elimination of salts, the blood of, for example, marine teleosts being not much more concentrated than that of freshwater species (i.e., the blood is hypotonic). As the fish kidney produces a hypotonic urine the salt which enters osmotically must be disposed of by other means. Marine fish (*71*) swallow large amounts of sea water and both water and salts are absorbed from the intestine. The excess salts, i.e., Na^+, K^+, and Cl^-, are excreted extrarenally. Mg^{2+}, Ca^{2+}, SO_4^{2-}, and PO_4^{3-} leave in the urine. Also only part of the water is excreted in the urine. Thus less well developed kidneys result and some may be without glomeruli and the tubules may be very short. [An aglomerular kidney must function by tubular secretion. Forster and Bergland (*76*) showed that the urine of the aglomerular teleost *Lophius americanus* contained a hundred times as much Mg^{2+} as the plasma.] The amount of urine produced in a marine teleost is small, e.g., 2.5–4 ml./kg./24 hours in toadfish and sculpin (*77*) compared with 200–400 ml./kg./24 hours in the freshwater gar pike (*71*).

Excretion of salt via the gills has been demonstrated (*78*). In the eel Cl^- is actively transported outwards when the animal is in sea water. In fresh water there was no outward transport of Cl^-. *Fundulus heteroclitus* (the killifish) can live in sea or fresh water. It is suggested that adaptation to the new environment is brought about either by active absorption of chloride from fresh water, or secretion of chloride into sea water. Pettengill and Copeland (*79*) suggest that the two functions of absorption and secretion are performed by the same cells in the afferent side of the gill filaments. In sea water these glandular cells have clear distal vacuoles containing chloride, but in fresh water the vacuoles disappear and the chloride is concentrated in a distal band of cytoplasm. This view is supported by the fact that injection of sodium chloride into the gut of freshwater-adapted killifish causes vacuoles to appear in the cells. Thus the ion regulation is set in motion by increased chloride in the blood and probably not by an external rise in salinity. It is interesting with regard to the mechanism of ion absorption that in the sea-water-adapted fish phosphatase is concentrated round the distal vacuoles while in the freshwater-adapted fish the distribution is throughout the cell. These results, however, should be interpreted with caution as a freeze drying technique was not used and, under the experimental conditions used, artifacts of fixation would be expected (*80*).

In fish, for example eel and salmon, which naturally spend part of their lives in the ocean and part in fresh water, the animals must switch from one mechanism to another according to the environment. For example *Anguilla vulgaris* (the European eel) has hypotonic blood in the ocean; it swallows sea water and secretes salt via the gills. In fresh water the glomerular kidneys produce a dilute urine, salt is replaced from the food, and the skin has a low permeability to salt and water.

The elasmobranchs are largely marine and the blood is always hypertonic (*81*). The salt concentration of the blood is only 41–47% of the total and is of the same order as that of freshwater teleosts. Retention of enormous quantities of urea makes the osmotic concentration up to that of sea water. Thus water is retained and a hypotonic urine can be excreted. The urea is retained by the animal having gills and skin which are relatively impermeable to it and presumably by reabsorption from the kidney tubule. Smith showed a 100% increase of urea in going from a freshwater species to a marine one. The output of urine is low in marine forms and contains Mg^{2+}, PO_4^{3-} and SO_4^{2-}.

The marine elasmobranchs have no great power of adaptation to changes in salinity.

Beadle (*36*) points out that analysis of the blood of the coelacanth, *Latimeria* will be of great interest. This is a marine animal with freshwater ancestry and it will be interesting to see if it has coped with the marine environment by retention of urea together with isotonic or hypertonic blood as in the elsamobranch or by the development of a mechanism for hypotonic regulation as in the marine teleost.

An interesting case of osmoregulation and balance in arctic fish is reported by Scholander *et al.* (*82*). Bottom-living arctic fish in Northern Labrador are permanently supercooled by almost 1°, i.e., their blood temperature is 1° below its freezing point and would freeze if seeded with a crystal of ice. It is only by this means that they are able to keep their ancestral hypotonicity. They also point out that these animals represent a case where "evolution has pushed life into a permanent physical metastability. A return to the stable physical state would be fatal." In contrast, the shallow-water arctic fish, which would readily be seeded by ice, double their osmoconcentration in winter which makes them almost isotonic with the sea water, and so freeze proof.

The possible endocrine control of balance has been investigated in the fish [review by Fontaine (*83*)]. It is known that hormones of the neurohypophysis affect the balance of salts and water in higher vertebrates (see later) and these hormones are present in the neurohypophysis of teleosts. Injection of teleost and mammalian hormones into teleosts has given no positive effect (*84*). The results of hypophysectomy have been conflicting but Burden (*85*) provides convincing evidence of the failure of hypophysectomized, sea-water-adapted *Fundulus heteroclitus* to survive in fresh water. The injection of pituitary extract enabled the hypophysectomized animals to survive. The pituitary extract from a strictly freshwater species was less effective and that from a marine species had no effect. The hypophysectomy had no effect on the cytology of the chloride-secreting cells in the gills but caused a reduction in the mucous cells. Thus the pituitary of *Fundulus heteroclitus* secretes an unknown factor affecting salt balance of the fish in fresh water, but the mechanism is unknown.

Histological changes in the neurohypophysis have been reported when salmon go from fresh to salt water and vice versa and in marine teleosts exposed to changed salinity. Also, there have been reports on the importance of thyroid in salinity tolerance but other evidence conflicts with this (reviewed by Fontaine). Thus the indirect control of balance by hormones is far from clear in the fishes.

b. Cyclostomes. The hagfishes (Myxinoidea) are entirely marine and the lampreys (Petromyzontia) either leave the sea to breed in rivers or are constantly in fresh water. The blood of *Myxine* is almost isotonic but the animal has more power of ionic regulation than the tunicates (*62*). Experimental data on the petromyzonts is inadequate, but Beadle (*36*) says there is little doubt that *Petromyzon marinus* is like the marine teleost in having a hypotonic blood in sea water. *P. fluviatilis* excretes a hypotonic urine in fresh water and actively absorbs chloride, thus resembling the freshwater teleost.

It has recently been shown by McFarland and Munz (*85a*) that the blood of *Polistotrema stouti* (the Pacific hagfish) is isotonic to the external medium. It resembles other vertebrates in the ratios of ionic constituents but is unique in its isotonicity and in having its osmotic constituents almost entirely ionic, i.e., no urea.

Morris (*85b*) discusses the osmoregulation of the lamprey (*Lampetra fluviatilis*). He showed that in sea water the mechanism is similar to that found in marine teleosts. The lamprey swallows sea water and absorbs a solution containing a high proportion of monovalent ions into the blood. The chloride is presumably secreted extrarenally by the chloride excretory cells in the gills. The rate of extrarenal water loss is high and the urine output is negligible but the urine is hypotonic to the blood. The external surface of the lamprey is much more permeable to water than is that of the marine teleost but there is evidence for a changing water permeability of the lamprey during its spawning migration. Morris suggests that lampreys have a freshwater ancestry.

c. Amphibia. The modern Amphibia are a group of animals showing a gradation from a purely freshwater form to a nearly terrestrial one. The frog, on which most of the work has been done, lies about the middle of the water-to-land range.

The sites involved in water and salt regulation are the skin, kidneys, and bladder. The blood is hypertonic to the pond water and similar to that of teleosts and most freshwater invertebrates and the urine is hypotonic to the blood. Thus the frog has problems in common with the freshwater fish, i.e., maintenance of hypertonicity of the blood and coping with water which enters osmotically. The kidney, as in other freshwater animals, is, therefore, an organ for the removal of water and the hypertonicity of the body fluid is maintained by active absorption of ions from the renal tubule. Unlike the fish, the skin has no scales and is highly permeable to water which enters continuously with the osmotic gradient and an efficient kidney producing a hypotonic urine is therefore a necessity.

The skin is also permeable to ions and actively absorbs Na^+ (and when chloride-depleted, Cl^-) from fresh water. Ussing (*86*) showed that the active inward transfer of Na^+ uses energy derived from metabolic oxidation [see Ussing (*11*)]. This mechanism is obviously functionally analogous to the absorption of ions by the gills of freshwater fishes.

It is probable that modern Amphibia are unique among vertebrates in developing a secondarily permeable skin. It is generally accepted that the rhipidistian fish ancestral to the tetrapods had an impermeable skin and scales and that the scales tended to be lost by the early Amphibia. But the line which gave rise to the tetrapods probably never had a permeable skin since this would have made it essential that they remain in a moist environment in order to prevent desiccation. Also, if oxygen could penetrate the skin easily, there would have been a tendency for the primitive lungs not to develop further and possibly even to degenerate.

When a frog is out of water it rapidly loses water through the skin and the blood osmotic pressure rises and a smaller volume of more concentrated urine is then produced (*87*, *88*). As water is not actively absorbed from the tubule the urine does not become hypertonic. The frog also becomes depleted of salts as these are also lost via the urine. After two hours out of water, urinary output may be almost zero. When a water deficient frog is replaced in water it does not drink but water enters rapidly through the skin. Intake is increased while the deficit remains.

A water excess can be induced experimentally by intraperitoneal injection of water (*88*). Intake via the skin continues unchanged, but urine output is modified and diuresis results. There appears to be no lag before the onset of diuresis. The recovery from desiccation by skin uptake is more rapid than recovery from excess by diuresis.

Thus balance in the frog is the result of the net entry of ions and water via the skin and loss via the urine.

There is good evidence for hormonal control of balance in the frog (*36*). The skin and renal tubules are affected by the hormones of the neurohypophysis. The first effect is the "water balance" effect peculiar to the less aquatic Amphibia. Injection of the hormones in the oxytocic fraction from the neurohypophysis causes an increased uptake of water through the skin of the intact frog. Also in isolated frog skin it causes increase in the passage of water and also in the total Na^+ flux. The second effect is the antidiuretic effect similar to that in mammals. This effect reduces glomerular filtration, probably by constriction of the afferent arterioles, and increases tubular reabsorption of water. Under extreme conditions the urine produced is almost isotonic. In *Bufo* and *Rana* there is reabsorption of water from the bladder when the animal is desiccated and this reabsorption is increased by neurohypophyseal hormones. There is some evidence that the stimulus for release of the hormones is an increased osmotic pressure or salt content of the blood (*89*).

The evidence for the hormonal control of the excretion and uptake of ions is not so satisfactory. Sawyer (*89*) is of the opinion that the mechanism for ion regulation is separate from that for water balance and though it may involve neurohypophyseal hormones it also involves those from the adrenal cortex.

The more terrestrial the amphibian the greater is the hormonal effect, for example in the purely aquatic forms *Xenopus* and *Necturus* the "water balance" effect is small and there is no antidiuretic effect (*90*). The strongest response is found in the almost terrestrial toads. An interesting sequence is shown in *Bufo* where the early tadpoles show no hormonal effect but it gradually develops during larval life (*91*).

Thus it is possible to suggest that the neurohypophyseal control of water balance evolved in parallel with the transition to terrestrial life. The antidiuretic response is obviously of very great importance to purely land forms while the "water balance" effect is peculiar to the Amphibia.

The interesting but unknown factor is the function of the neurohypophyseal hormones in the fishes. It seems reasonable to suggest that they already had some function and were used for new purposes by various groups as new situations arose, for example in the Amphibia. The high class specificity of the hormones (*92*) is of interest in this connection. The hormone from the same species is most effective in causing a response and this may be the oxytocin or vasopressin fraction of the secretion. In the frog oxytocin is more effective than vasopressin. Thus perhaps the protein substance of the neurohypophysis produces a series of polypeptides which have been exploited in different ways by different groups of animals though there may be a basic type of response. For example, the effect may be on smooth muscle as suggested by Ewer (*90*). In *Anura* the action is on the renal blood vessels and in mammals oxytocin ultimately affects the pregnant myometrium. Ussing (*93*) suggests that the hormones act on frog skin and renal tubules by causing dilatation of pores.

In the desiccated frog there appears to be no behavioral mechanism directing it to water, which it finds by chance (*88*). However in other Amphibia, for example *Triton*, such a mechanism appears to exist.

d. Reptiles. One of the main problems of the terrestrial animal is conservation of water. In the reptiles this has been achieved by the presence of scales which reduce surface evaporation, by the production of a small amount of urine, and by the development of the cleidoic (protected) egg or viviparity. There is, however, no evidence for the production of a urine hypertonic to the blood in the reptiles which have been investigated, for example, turtle and tortoise (*71*).

Many reptiles, for example, *Thamnophis* (garter snake), *Chrysemys* (freshwater tortoise), *Anolis, Uta* (lizards), and alligator show a water deficit on confinement without water. The main water loss is presumably from the lungs. The deficit can be made good by drinking which is rapid, i.e., like the dog.

An excess water load can be given to *Thamnophis* by intraperitoneal injection and this is then eliminated by the expulsion of a liquid from the cloaca instead of the usual semisolid mass. The usual water turnover in *Thamnophis* is small. Some water is normally drunk each day but Adolph (*88*) suggests that water balance could be maintained by water produced by metabolic oxidation.

In the alligator the urine flow is 0.4–1.2 ml./kg./hr.

Recently it has been shown (*93a*) that in the lizard, *Trachysaurus rugosus*, Pitocin and Pitressin cause antidiuresis and that adrenalectomy causes a rise in plasma K^+ and no change in plasma Na^+.

e. Birds. Birds can produce a hypertonic urine and the renal tubules have the thin loop of Henle.

The blood of the hen was shown by Korr (*94*) to be equivalent to 0.925–0.94% NaCl and the uretal urine equivalent to 0.95–1.1% NaCl. During water deprivation the urine concentration of the hen increased. Uptake of water from the urine was also increased by injection of antidiuretic hormone. The urine may be a mass of uric acid crystals as water can be absorbed from the cloaca (*95*).

Schmidt-Nielsen and co-workers (*95a*) have recently shown extrarenal salt excretion in birds. When the cormorant is given a salt load it excretes a highly hypertonic (to the blood) liquid which drips out of the nares and collects at the tip of the beak. The secretion is practically only sodium chloride (500–600 mmole NaCl). The secretion is also stimulated by sucrose, indicating that the response is to general osmotic conditions. When the cormorant is fed fresh fish its water intake is adequate.

f. Mammals. A great deal of work has been done on balance in mammals.

The primitive mammals were terrestrial and therefore one of their problems was water conservation. This is achieved as in birds by the adaptation of the primitive water-excreting kidney to an organ able to produce a hypertonic nitrogen-containing urine from which water is reabsorbed as well as salts. The main loss of water under normal conditions is, however, via the urine. In a man of 70 kg. it is 600–2000 ml./day.

The skin is lacking in scales but is not very permeable to water and the so-called insensible perspiration amounts to 350–700 ml./day. The skin, however, contains sweat glands which secrete water, salts, and other substances and this loss can be of great importance in over-all balance, especially under desert conditions. The daily water loss in sweat in man is 50–4000 ml. Other glands, for example, tear and salivary, actively secrete various substances but can be neglected in balance studies.

Water loss is also effected by the lungs where air is saturated with water vapor. In man this loss amounts to 350–400 ml./day. The amount lost via the feces is small (50–200 ml./day). Thus under normal conditions the main loss is via the urine, but under desert conditions more may be lost in sweat.

Again balance has two aspects: (*a*) the permeabilities of the various external membranes, and (*b*) the over-all effect balancing intake with output mediated by hormonal, muscular, nervous, and behavioral activity.

Under normal conditions intake of water and salts is periodic whereas output of urine is continuous. Therefore the animal fluctuates about the point of balance.

The control of balance has been studied by inducing excesses and deficits in dog and man (*88*).

Excess water can be given to dogs by gastric administration. After a latent period this leads to greater water loss than usual (diuresis). The increased water output is only via the urine. The normal balance is recovered after a few hours. Administration of water by different routes does not have the same result. Intravenous injection causes a delayed diuresis which is less intense and prolonged. Subcutaneous water causes no diuresis while the response to water given by rectum is prompt. The results with man are basically similar. These different responses may be due to changes in the rate of plasma protein dilution in the different methods which would affect extra/intra cellular distribution of water. Also it should be borne in mind that water diuresis can only start when any antidiuretic hormone in the blood has been removed. Because of this a lag may result before the onset of diuresis.

A water deficit can be induced in dogs by witholding water. During the deficit, dehydration of the tissues occurs as a minimal output of urine is maintained. However, all the tissues do not lose water equally and not in proportion to their normal water content. The plasma concentrates more than twice as much as the whole body. Thus the plasma possibly protects the tissues to some extent by taking the strain of the dehydration though this relief to the tissues is, of course, only temporary as the reduced plasma volume ultimately causes circulatory failure.

In water deficiency urine production is reduced. This antidiuresis due to dehydration is caused by stimulation of reabsorption brought about by the release of antidiuretic hormone from the neurohypophysis. The site of action of the hormone is thought to be the distal tubule and the reabsorption is concerned with that part of the filtered water not passively reabsorbed (as a result of ion uptake) in the proximal tubule (*96, 97*). The part played by electrolyte excretion during antidiuresis is not clear. The active antidiuretic principle is now known to be vasopressin but it must be borne in mind that stimulation of the neurohypophysis by dehydration causes release not only of vasopressin but also oxytocin. Oxytocin may cause changes in sodium and potassium excretion (*98*).

Verney (*99*) suggested that the stimulation of osmoreceptors in the brain as a result of raised osmotic pressure of the blood caused impulses to be sent to the neurohypophysis causing release of antidiuretic hormone. Jewell and Verney (*100*) have now shown that the osmoreceptors are localized within the anterior hypothalamus and perhaps within adjacent parts of the preoptic area. They also suggest that the functioning of the osmoreceptors may be dependent upon the integrity of the nervous connection with the thalamic paraventricular nucleus [reviewed by Zotterman (*101*)]. This fits in with the observations of Andersson and McCann (*102*) that electrical stimulation of the anterior hypothalamus led to inhibition of water diuresis in goats (while at the same time causing drinking).

When a dehydrated dog is allowed access to water, the deficit is restored in a few minutes and the speed of drinking and its sudden cessation are dramatic. In this case the amount required for balance is drunk before the water is absorbed from the gut and so the control of balance in the dog must in some way be linked to "thirst." Adolph (*88*) drew the following conclusions about "thirst" in the dog:

(i) Water is metered as it passes the mouth and pharynx and this removes the urge to drink for about 30 minutes.

(ii) Even water which does not pass through the throat removes the urge to drink once it has been absorbed.

(iii) At least two satisfactions of "thirst" occur: a temporary one in the throat, and a more lasting one following distribution of water to the tissues.

Thus there is a close correlation between the deficit and the amount of water drunk. In man a rather different situation exists as a dehydrated man will not voluntarily make up the deficit when offered water but will remain somewhat dehydrated for several hours. This may be a psychological phenomenon and is not understood.

The theory that "thirst" is, at least partly, caused by cellular dehydration has been put forward by many workers [review by Andersson (*103*)]. Wolf (*104*) suggested that it was due to the stimulation of osmoreceptors similar to those regulating the activity of the neurohypophysis. Andersson and McCann (*102*) showed drinking in goats following electrical stimulation of the anterior hypothalamus (see above).

It seems possible that "thirst" is also related to food intake; Lepkovsky *et al.* (*105*) have shown that in rats fed without water the gastric water content is approximately 49% and the same as that in rats fed with water. Thus water from the tissues is used to maintain the appropriate percentage of water in the stomach. "Thirst" may therefore be stimulated by dehydration due to food intake.

Greer (*105a*) has given suggestive evidence of a primary "drinking center" in the hypothalamus of the rat by implanting electrodes and stimulating the hypothalamic region.

In his recent book Wolf (*105b*) reviews older and more recent views on thirst. He supports the view that the sensation of thirst originates in osmoreceptors located in a "thirst center" in the hypothalamus which overlaps the center of regulation of the antidiuretic hormone of the neurohypophysis. He considers that the dehydration of such osmoreceptors would be paralleled by that of general body tissues which would include the membranes of the oral cavity. Thus there exists a pathway for the setting up of a conditioned reflex whereby local dryness of the mouth and throat (however caused), or osmotic or chemical stimulation of their membranes, elicits thirst. This conditioned stimulus may act through sensory nerves from the mouth and throat which join a primitive thirst reflex whose primary afferents (attached to the osmoreceptors) lie in the central nervous system.

Wolf points out that the above theory is not entirely satisfactory. He shows that in certain conditions, e.g., in fasting dogs allowed free access to water, thirst is augmented by cellular hydration. This might be explained in terms of an "accommodation" in the osmoreceptors (*105c*); or alternatively Wolf suggests that excessive swelling of the osmoreceptors (as in hypotonic thirst) might stimulate the afferent nerves as does shrinking (in "normal" thirst).

Radford (*105d*) working on rats, came to essentially similar conclusions about the afferent pathways governing drinking: (*a*) oral receptors sensitive to the concentration of mouth fluids; (*b*) gastric receptors sensitive to distention of the stomach; and (*c*) receptors sensitive to the concentration or volume of body-fluid, possibly the same as those concerned with water excretion.

Very pronounced water and salt deficits are obtained in man living under desert conditions where rise in body temperature causes sweating and hence loss of water and salts via the sweat. The dog loses water by panting and hence its blood concentration rises more rapidly than that of man. The results of water loss by sweating are similar to those obtained by witholding water at normal temperatures (*106*).

The study of animals which normally live in the desert (*107*) shows that they can, broadly speaking, be divided into two groups: (*a*) animals which expend water for heat regulation, e.g., camel, also man and dog; (*b*) animals which do not expend water for heat regulation, e.g., desert rodents such as the kangaroo rats (*Dipodomys*).

The kangaroo rats do not need drinking water or moist food and the metabolic water is carefully used. Evaporation from the body is only half that of the white rat and the urine is twice as concentrated due to high tubular reabsorption of water. The concentration of antidiuretic hormone in the pituitary and in the urine is higher than in the rat and dog. The feces are very dry. But the animals are not able to withstand an exceptional degree of dehydration.

The kangaroo rats are burrowing and nocturnal animals and so avoid an increase in body temperature due to the heat of the day.

Among animals which expend water for heat regulation, man and dog have already been discussed. The camel, a normal inhabitant of the desert, has been investigated by Schmidt-Nielsen *et al.* (*108, 109*). It shows certain adaptations to its desert environment and can tolerate a water loss corresponding to 30% of its body weight, as compared with other mammals which die from circulatory failure when the water loss is 12%. Unlike other mammals it eats normally until it is very severely desiccated. It is not known whether the water found in the stomach is ingested or secreted. The output of urine is also low (0.5–1 liter/day when fed on dates and hay), and there is a low water loss in the feces. When dehydrated there is low evaporative water loss. In summer the diurnal variation of temperature in the camel deprived of water is about 6°. In winter or in summer with water the variation is about 2°. Thus heat is stored in the body instead of being dissipated by the evaporation of water. The dehydrated camel can drink to rehydration in about 10 minutes. It is suggested that the peculiar oval red cells of the camel may be less of an obstruction in capillary circulation during dehydration.

The blood of those mammals which have become secondarily marine is only slightly more concentrated than that of the terrestrial mammals and consequently it is hypotonic to the sea. The urine however is hypertonic to the blood and combined with a fish diet this is sufficient to maintain the blood hypotonic to the environment. In marine mammals feeding on marine invertebrates (e.g., walrus and whalebone whales), where the ions in the food are more concentrated than in the blood, the water balance problem is more acute. Cutting down the water loss via the lungs and feces, however, appears to be sufficient to maintain balance (*35*).

As mammalian urine is hypertonic to sea water, mammals should be able to extract water from the ocean. In man utilization of sea water is limited by the gastrointestinal tract and not by the kidneys (*110*). The kangaroo rat can however extract water from sea water and so can the Australian marsupial *Setonyx brachyurus* (*111*).

Recent papers by Prentiss *et al.* and Wolf *et al.* (*111a,b*) show that the cat can meet its water requirement from a diet of fish or meat and that it can survive by drinking sea water.

Wolf (*105b*) mentions experiments on anesthetized dogs with large osmotic loads of sodium salts in which a nasal secretion hypertonic to the plasma is produced, cf. cormorants (Section II,C,8,*e*).

Kuno (*111c*) shows that in mammals with small mouths (i.e., with insufficient mucous membrane to control body temperature by evaporation from the membrane) which do not pant and do not have sweat glands, e.g., mouse, rat and guinea pig, saliva is used as a substitute for sweat. Following copious salivation the animal distributes the saliva over the body. Kuno also considers heat loss in the elephant, an animal with a very large body and small mouth, without sweat glands and showing neither panting nor copious salivation. He observed that on warm days elephants suck water into the trunk and spray it over the body. In the absence of water, saliva may be used in the same way.

Salt balance is maintained by intake by the gut and output via the urine and sweat and other glands. In desert conditions the loss in the sweat is very great (in man) but under normal conditions the main loss is in the urine.

Selkurt (*112*) reviews sodium excretion by the kidney. He suggests that tubular reabsorption is regulated by intrarenal and extrarenal mechanisms. These correspond to the membrane effect and the coordinating control exerted by hormonal and nervous activity.

It is suggested that the urine remains isotonic in the proximal tubule and thin segment and 80–90% of the filtered water is reabsorbed as a result of active uptake of solute (mainly NaCl and $NaHCO_3$) in the proximal tubule (*97, 113, 114*). During water diuresis, 10–20% of the water of the glomerular filtrate can be excreted with negligible salt content, thus suggesting that Na salts are reabsorbed in the distal tubule. It may be better however (*112*) to view sodium reabsorption as a changing fraction of the amount filtered rather than a constant fraction of proximal/distal excretion.

A sodium load can be given to the dog by intravenous infusion of hypertonic NaCl. Under such conditions the glomerular filtration rate (GFR) may change, i.e., the volume filtered per unit time changes. But a change in GFR is not always found and even in these cases the concentration of sodium in the urine rises sharply. This suggests (*112*) that the sodium transfer system has a limiting absorptive capacity which automatically helps to buffer the blood. A reduced sodium load can be given to the tubules by reducing GFR experimentally. Then the reabsorption may be complete and the urine become free of Na^+.

When a substance is ingested or injected into man which is excreted in the urine, e.g., nonelectrolytes such as mannitol, an increased urine flow results. This is termed osmotic diuresis. During such osmotic diuresis by mannitol, glucose, sucrose, urea, xylose, or creatinine, increased excretion of Na^+ occurs, though less sodium is lost than water. Wesson and Anslow (*113*) suggest that retention of water in the tubule as a result of the osmotic effect of the nonelectrolyte dilutes the Na^+ concentration in the urine below that of the plasma and so reduces the rate of Na^+ reabsorption.

Morel (*115*), working on potassium excretion, suggests that all the K^+ in the glomerular filtrate can be reabsorbed in the proximal tubule and that the K^+ in the urine is secreted by the distal tubule.

The role of the hormones of the anterior pituitary and the adrenal cortex in electrolyte excretion is not yet clear though a great deal of work has been done on the subject [reviewed by Nabarro (*116*)]. There is evidence that growth hormone causes Na^+, K^+, and nitrogen retention in hypophysectomized or adrenalectomized rats (*117, 118*). This mechanism presumably does not involve the adrenal cortex and is obscure.

Patients with adrenal insufficiency and adrenalectomized dogs showed salt depletion as a result of increased sodium excretion in the urine. The GFR is reduced and the normal diurnal variation of excretion is upset. It is suggested (*116*) that the adrenal steroids act on the sodium reabsorption process in the renal tubule. The reduced GFR is a protective mechanism to prevent further sodium loss. Adrenal insufficiency also results in potassium and nitrogen retention. Nabarro attributes this to the low GFR and to a loss of enhanced potassium excretion and to increased cell catabolism.

However, the complete picture of the role of ACTH and the adrenal cortical hormones is not clear, though Selkurt (*112*) is of the opinion that the concept of a neurohypophyseal hormone aiding regulation of electrolyte balance can be universally entertained.

III. The Distribution of Diffusible Molecules within the Organism

A. HISTORICAL: INTRODUCTION

In the preceding section it has been shown that organisms can maintain a steady-state composition which can be quite different from that of their external environment. Within the organism there can further be marked differences in composition from tissue to tissue correlated with the differences in function of these tissues. In what follows a comparative treatment of the distribution of diffusible substances within the organism will be presented.

The historical background to present knowledge of the distribution of water and electrolytes within the organism is given in the excellent review of Manery (*119*). Briefly (references in Manery) it was early realized that the mineral elements were not uniformly distributed throughout the organism. Thus, Liebig in 1847 found that muscle was rich in potassium and poor in sodium whereas blood, from which the muscle was formed, contained much sodium and little potassium. Later (1891), Halliburton concluded that solid tissues were rich in potassium phosphate whereas the fluids (except milk) contained sodium chloride. These distribution studies led to an attempt at their explanation. Measurements of the electrolyte and nonelectrolyte permeability of various cell types (Overton, Höber) and especially of the erythrocyte (Jacobs) showed, in general, a low electrolyte but a higher nonelectrolyte permeability. Thus it could be suggested that the nonelectrolytes were passively distributed between cell and surrounding fluid. The cations were, it was thought, confined to either cellular or extracellular fluid by their impermeability. The smaller anions however were considered to be passively distributed (at least in the red blood cell where their permeability was high) but in accordance with the prevailing Donnan potential due to fixed anions (Peters and van Slyke).

This scheme has had to be drastically modified as a result of the experimental work of the last 20 years. The first of these studies was that of Huf (*120*) who demonstrated that a frog skin separating two "Ringer" solutions was able to transport inwardly both water and (more important for our purposes) chloride ions. As the two Ringer solutions were initially identical in composition, chloride ions had been moved against the chemical gradient. Krogh (*121*) showed that living frogs and also Crustacea were able to take up chloride ions against a steep concentration gradient. (Ion movements in amphibia skin have been intensively studied in recent years, especially by Ussing. The fundamental process seems to be an inward active transport of sodium, chloride ions following passively.) Later Harris (*122*) showed that human red blood cells were able to accumulate potassium ions against a chemical gradient. Thus it could be suggested that the cell membrane actively controlled ion distributions by salt transport against the chemical gradient. Such a view was necessitated by the demonstration of the permeability of many cell membranes to isotopically-labeled cations (*123*), forbidding explanations of ion gradients in terms of passive impermeabilities.

These two types of study have now been integrated into the concept of the active participation of cell membranes in determining ion distributions, in the face of a definite permeability of these membranes (*121*). This will be discussed in the following pages. The capabilities of individual cells in determining distribution gradients will first be considered and then the properties of cells organized in tissues.

B. Balance Across the Cell Membrane*

Data on the distribution of substances across cell membranes are most easily obtained when the cell can be separated from its surrounding medium and the two phases separately analyzed. This is only possible for preparations of isolated cells which are unfortunately rare. Suitable systems (see Table I) are those of unicellular organisms: bacteria, protozoa, yeasts, and certain plant algae; also metazoan egg cells and sperm, and blood cells. Certain crustacean nerve and muscle cells are large and can be dissected out and analyzed separately from their surrounding tissues. Ascites tumor cells occur isolated in the ascitic fluid of the afflicted animals and are proving to be an extremely useful experimental preparation. Methods are being developed for the preparation of isolated cell suspensions from whole organs (*125*) but no data on their capabilities for accumulation are available. These are awaited with much interest.

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* The subject has been reviewed by Manery (*119*) and Harris (*124*).

If the cells concerned cannot be isolated from adjacent tissues in a viable form, then only indirect estimates as to gradients across the cell membrane can be found. Some such data are available, however, and can prove very valuable. The method used is to determine the concentration of the component Q in the whole tissue. Then, if the relative volume of the tissue occupied by the cells alone is known and also the concentration of Q in the extracellular fraction of the tissue can be estimated, Q in the cell can be calculated. The extracellular fraction is, in suitable preparations, merely the interstitial fluid which is presumed to be in equilibrium with the lymph and to have the same composition (a protein-free blood ultrafiltrate is used for organisms which have no lymphatic system). The difficulty of the technique and the assumptions made in the derivation of the intracellular value of Q are clear. Data on the intracellular composition of vertebrate muscle has been obtained in this way. This information, together with that derived directly, is collected in Tables I and II as the ratio of the intracellular concentration of the component Q at equilibrium to that in the medium, calculated from the published data. In addition, rather more qualitative information on the distribution of minerals within a tissue can be found by microincineration studies (*126*). For hydrogen and hydroxyl ions, pH measurements give a measure of the gradient across the cell membrane. An excellent and comprehensive review of methods and results of pH studies is given by Caldwell (*127*). In many cases the distribution of hydrogen ions does not accord with the Donnan theory and active transport systems have been involved. These ions are always in low concentration (about $10^{-7}\,M$) in cells (with the exception of acid-secreting cells), and thus the maintenance of a pH gradient, although closely linked to metabolic processes, does not in general require much of the available energy of the cell (again with the exception of the acid-secreting cells). The distribution is further complicated by transport and accumulation as metabolic acids and bases. They are thus excluded from Table I.

The data in Table I uniformly bear out the century-old views of Liebig and Halliburton above—i.e., the interior of the cell is rich in

TABLE I
Ionic Gradients across Cell Membranes

Organism	Cell studied	External medium	Intracellular/extracellular concentration ratio				Reciprocal of ratio			References
			Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	PO_4^{3-}	
Bacteria										
Escherichia coli	whole animal	2×10^{-4} *M* K^+ in culture medium	1	up to 1000						*128*
Alcaligenes faecalis	whole animal	1×10^{-4} *M* K^+		up to 100						*129*
Yeast										
Saccharomyces	whole animal	3×10^{-4} *M* K^+ in glucose buffer		up to 2000						*130, 131*
Protozoa										
Spirostomum	whole animal	fresh water	0.9	10.8			7.6			*132*
Metazoa										
Sea urchin (*Paracentrotus*)	unfertilized egg	Roscoff sea water	0.11	21	0.36	0.2	7	5		*133*
Holothurian (*Caudina*)	blood cell	plasma	0.45	15	0.65	0.7	4.2	0.23		*134*
Cephalopod										
Loligo	nerve (resting)	artificial sea water	0.09	31			7.9		0.06	*135, 136*
	nerve (stimulated)	artificial sea water	0.3	23			6.4		0.05	*135, 137*
Sepia	nerve	artificial sea water	0.08	32			15		0.06	*135*
Crustacean										
Carcinus	muscle	plasma	0.09	9.2	0.3	0.7	9.9			*138*
Amphibian										
Frog	muscle	plasma	0.03	58	2.5	12	50			*139*

Reptile								
Tortoise	red blood cell	plasma	0.15	13.6				*12*
Birds								
Duck	red blood cell	diluted plasma	0.05	18				*140*
		no oxygen	0.06	22			1.33	*140*
Mammals								
Rat	muscle	plasma	0.02	24	0.61	11	24	*139*
Cat	red blood cell	plasma	0.90	1.7			1.33	*141*
Dog	red blood cell	plasma	0.88	2.1			1.44	*141*
Ox	red blood cell	plasma	0.69	6.8			1.28	*141*
Sheep	red blood cell	plasma	0.61	9.6			1.49	*141*
Rat	red blood cell	plasma	0.18	23.0			1.44	*141*
Horse	red blood cell	plasma	0.11	25.0			1.27	*141*
Rabbit	red blood cell	plasma	0.15	25.4			1.38	*141*
Baboon	red blood cell	plasma	0.15	30.8			1.48	*141*
Man	red blood cell	plasma	0.16	27.4			1.44	*141*
Man	red blood cell	refridg'ed 6 days	0.42	7			1.2	*141*
Rabbit	white blood cell	plasma	0.5	19			1.2	*142*
Horse	white blood cell	plasma	0.75	4.4			1.5	*142*
Mouse	ascites tumor cells	ascitic fluid	0.39	33			1.8	*(142a)*
Plants (single celled)								
Valonia	(sap)	sea water	0.18	42	0.14		0.97	*4*
Halicystis	(sap)	sea water	1.1	0.5	0.75		0.96	*4*
Chara	(sap)	brackish water	2.4	6.3	2.9	2.4	0.325	*4*
Hydrodictyon	(sap)	pond water	3	4000	1.6		0.02	*4*
Porphyra	(cytoplasm)	sea water	0.1	40	0		6–7	*(142b)*

TABLE II
GRADIENTS OF AMINO ACIDS ACROSS CELL MEMBRANES

Organism	Cell studied	External medium	Intracellular/extracellular concentration ratio		References
Bacterium					
Staphylococcus aureus	(whole animal)	buffered salts	Proline: 1670; Glutamic acid: 324; Lysine: 19.6; Tyrosine: 8.65; Alanine: 19.7; Glycine: 81.5		*143*
		buffered salts plus inhibitor (dinitrophenol)	Proline: 9.9; Glutamic acid: 2.8; Lysine: 63; Tyrosine: 5.36; Alanine: 19.6; Glycine: 92.2		*143*
Mammal					
Mouse	ascites tumor cells	Ringer solution (+ 2 mM amino acid)	Glycine: 10; Proline: 2.5; L-Alanine: 18.6; D-Alanine: 6.8; Lysine: 3.5; Glutamic acid: 15 (av.);	α,γ-Diaminobutyric acid: 18.7; α-Amino-γ-methylaminobutyric acid: 5.5; γ-Aminobutyric acid: 1.05; α-Aminoisobutyric acid: 4.8	*144*
Rabbit	Muscle	Plasma	Alanine: 2.1; Arginine: 1.4; Aspartic acid: 4.2; Glutamic acid: 3.3; Glycine: 9.2; Phenylalanine: 2.0; Threonine: 2.7; Valine: 1.5;	Histidine: 31; Isoleucine: 1.9; Leucine: 2.0; Lysine: 1.3; Methionine: 3.8; Proline: 2.2; Tyrosine: 3.7;	*145*
Cat	Muscle	Plasma	Histidine: 61	Glutamic acid: 13	*146*
Bird					
Chicken	Muscle	Plasma	Histidine: 179		*145*
Fish					
Cyprinus	Muscle	Plasma	Glycine: 36 Proline: 6.1	Histidine: 120	*147*
Crustacean					
Homarus	Muscle	Serum	Alanine: 19 Glycine: 43	Arginine: 486 Proline: 121	*148*

potassium and phosphate and poor in sodium and chloride in comparison with the extracellular fluid. Unfortunately, data are insufficient to permit generalization of this statement with confidence to include all cells. Thus, as will be emphasized in the next section, numerous insects have (atypically) reversed ratios of sodium to potassium in their blood. Information on the intracellular ion concentrations of these insects is lacking, but a hypothesis which will be outlined in the following pages predicts that these cells should be of high sodium content and low potassium content.

The explanation of these data poses immediately the twofold problem: What are the molecular mechanisms by which these transmembrane concentration gradients are maintained? What is the physiological role (if any) of the high potassium concentration and low sodium concentration in the cell?

Dean in 1941 (*149*) made the fruitful suggestion that the intracellular ion concentrations were maintained by cation "pumps," either pumping sodium ions out of the cell, pumping potassium in, or both. This hypothesis used the concept of active ion transport to reconcile the data on ion distributions with the developing view of the cation permeability of the cell membrane.

Few experimental data are available which would allow a decision to be made as to what type of pump is active. It may be of value, therefore, to consider the physiological role of such "pumps" at this point.

That it is probably essential for an ion-permeable cell to pump out at least one major ion species to maintain its equilibrium volume was pointed out by Maizels (*32*) and by Flynn and Maizels (*150*), and support for this view—i.e., that the fundamental role of these ion pumps is in *volume* regulation—has been increasing (*4*, *139*). The hypothesis may be enunciated as follows.

Consider a model cell in a marine environment. It is assumed that the cell is permeable to small anions and cations. However, the large anions must be assumed to be retained within the cell as being essential for cell metabolism and expensive to synthesize. The internal fixed anions impose a Donnan distribution on the diffusible ions, but it can readily be shown that even at the Donnan equilibrium there is still an osmotic excess within the cell which is thus in hydrodynamic disequilibrium. This effect will be enhanced since, further to conserve its energetic resources, it will be of advantage to the cell to retain its synthesized larger nonelectrolytes (in general, however, only small intracellular concentrations of such nondiffusible nonelectrolytes are found.)

The cell is thus forced to expend energy to maintain a constant volume in the face of this osmotic inflow. One method of maintaining a constant volume would be simply to pump out the incoming water. Another possibility exists, however, and that is to pump out some of the incoming ions. This reduces the concentration of ions inside the cell and the osmotic inflow of water is reduced. A simple formula can be derived which relates the concentration of diffusible ions, which must be "fixed" outside of the cell by the ion pump, to the concentration of nondiffusible internal anions and the total external salinity.

$$r = \frac{2B}{2B - [(n+1)A + P]} \tag{1}$$

$$\text{Fixed Na}^+ = \frac{(r^2 - 1)B - rnA}{r^2} \tag{2}$$

(In Eqs. 1 and 2: r is the Donnan ratio at equilibrium; B is the total external diffusible anion; A is the concentration of internal fixed anion; n is the valency of this anion; P is the concentration of internal nondiffusible non-electrolyte; and "Fixed Na^+" is the concentration of fixed Na^+ for equilibrium.) Thus both Donnan and hydrodynamic equilibrium can be achieved by the action of the ion pump. (The pumped out ion is, of course, not in Donnan equilibrium.) Calculations of the energy which would be required to achieve this equilibrium by a water pump or by a sodium pump, on the basis of typical values for the internal fixed anion concentrations and for cation and water permeabilities, show the sodium pump to be by far the most economical of energy. This is because cell membranes in general have a low cation permeability, but a high water permeability. The concentration of ion which is "fixed" outside the cell must always be at least as great as the (osmotic) concentration of fixed internal anion. Thus to fix a minor component of the external cation (for example, potassium or magnesium) would, in most cases, be insufficient to balance the intracellular anions. Furthermore, if the intracellular fixed anion concentration is high, the high Donnan potential will prevent chloride ions from crossing the cell membrane. This is equivalent to reducing the effective external chloride concentration, and again insufficient chloride will be available to balance the internal anion [for an alternative explanation for the absence of a general "chloride pump" see Conway (*139*)].

Thus hydrodynamic equilibrium can be maintained in spite of a significant intracellular fixed anion concentration, if the major external cation is (to an extent defined by Eq. 2) excluded, i.e., if there is a sodium pump. (A third possible mechanism is the maintenance of a high external nonelectrolyte concentration. A unicellular organism cannot alter its marine environment in this way but in the higher metazoa the extracellular fluid can conceivably be so altered.) There is, therefore, a plausible physiological role for the sodium pump which Dean suggested. Maizels has pointed out that a pump moving potassium inwards would exacerbate the fixed anion effect so that a simple potassium pump acting alone is unlikely. However, the possibility remains to be considered of both the outward sodium pump and the inward potassium pump acting as linked systems or otherwise. These pumps cannot be linked one-for-one if osmotic benefit is to be derived from them—but some other linkage is possible.

If only the sodium ion is actively regulated, the other ions will distribute themselves according to the potential which now arises from the combined effect of the intracellular fixed anion and the (virtually) fixed external sodium, and will thus lead to a Donnan-type equilibrium. The "Donnan ratio" r can be calculated from a knowledge of the internal fixed anion concentration and the external salinity (Eq. 1). Unfortunately, data from which the internal fixed anion concentration can be found are available in only a few cases: sea urchin egg (*133*), crustacean nerve (*151*), crustacean muscle (*138*), frog muscle (*139*), mammalian red blood cells (*152*). In the first four cases, the calculated Donnan ratios are in reasonable accord with those observed. In the case of mammalian red blood cells, the data from the low potassium group of cells (see Table I) agree with the theory, but the other cells, i.e., most red cells, are anomalous.*

For cells where no direct check on r is possible, a preliminary check of the theory can be made by comparing the transmembrane gradients of the (presumptively) passively distributed ions. From Table I (ignoring the values for plant cells which refer to sap rather than to protoplasm) it can be seen that the data for potassium and chloride are in reasonable accord with this assumption for all the cells other than certain of the mammalian blood cells, but again the paucity of data forbids any confident generalizations. In the case of cells large enough to admit an intracellular electrode, the Donnan potential can be measured directly, and its relation to the external and internal ion concentrations studied (*4, 124, 139*). In general, these studies confirm that a Donnan equilibrium is reached by the potassium and chloride ions, though some authors contest this for certain nerve and muscle cells [see discussion in Harris (*124*); also Altamirano and Coates (*153*) and the series of papers by Simon and Shaw (*154*)].

A recent study of ionic equilibria in lamellibranch muscle [Potts (*154a*)] weakens this generalization. Thus the potassium and chloride ions in the slow part of the adductor muscle of *Pecten*, the adductor and byssus retractor of *Mytilus*, and the adductor of *Anodonta* are all not in Donnan equilibrium with the ions of the corresponding blood. In the fast portion of the adductor muscle of *Pecten*, potassium and chloride equilibria are, however, governed by the Donnan relations. Also, Shaw (*154b*) provides further evidence that the potassium distribution in *Carcinus* muscle is very largely governed by the Donnan equilibrium.

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* Unpublished calculations by one of the present authors—W. D. S.

In the case of those red cells with a very high internal potassium content, the simple fixed anion hypothesis does not hold. A strict linkage of sodium exit and potassium entrance has been demonstrated for these cells (*155*). A recent study gives 3/2 (rather than the earlier value of 2/1) for the ratio of sodium ions transported outward to potassium transported inward (*156*). In refrigerated red cells the ion ratios are closer to those predicted by the simple fixed anion hypothesis (*141*).

Ascites tumor cells of the mouse are similar to the high-potassium red cells, in that the simple fixed anion hypothesis does not hold (*142a*). Hempling has shown that it is possible to separate here the potassium and sodium transport systems, so that the similarity in ionic distributions may not reflect a common mechanism in red cells and these tumor cells. Also, for rabbit white cells, where again the potassium concentrations are higher than those expected from a simple Donnan distribution (Table I), Elsbach and Schwartz (*156a*) have shown that a system exists for the active accumulation of potassium with little or no net extrusion of sodium. The potassium accumulation system is dependent upon an anaerobic glycolytic supply of energy.

In contradistinction to most of the cells listed in Table I, red cells have a low fixed internal anion concentration. The linked sodium-potassium exchange in the anomalous red cells is thus possibly a specialization which raises the potassium content of these cells to the levels found generally. Similarly, in plant cells where the cellulose wall prevents the cell volume from expanding, high potassium content (and presumably sodium pumps) are general but have no obvious volume-regulating role. It is suggested that in the course of cell evolution, cells have become adapted to a high cell potassium which has, indeed, become essential for protoplasmic activity (*139*). The low potassium-content red cells then become secondarily an anomaly on this viewpoint, having (possibly) lost their potassium pump. (It is necessary, however, to invoke here an unexplained effect of the potassium/sodium ratio on cell activity and it was the virtue of the fixed anion hypothesis that no such effect need be postulated generally.) In nervous tissues, the high Donnan potential has a physiological role in the transmission of nerve impulses (*157*). It may be useful to consider the unusual intracellular fixed anion isethionic acid of these cells (*151*) as a specialization to achieve a high Donnan potential. A linked sodium-potassium exchange would not have this effect. It is clear that information on the intracellular ion content of those organisms having a high blood potassium would be of critical importance in testing the fixed anion hypothesis. These data are awaited with interest.

In the case of the crab *Carcinus*, the concentration of the fixed internal anion (mainly free amino acids, taurine, and trimethylamine oxide) in the muscles, can be varied in response to changes in the external (blood) osmotic concentration—an adaptation for life in brackish water [Shaw (*157a*)].

Evidence for the presence of a sodium extrusion system in plant material is available from the work of Eppley (*157b*) on the plant *Porphyra*, a red marine alga lacking a central vacuole. Similarly, the external (plasmalemma) membrane of the vacuolated alga *Nitellopsis* has been tentatively identified as the site of a sodium extrusion system (*157c*).

Apart from this general cell property of the accumulation of mineral ions, recent studies (cf. Table II) make it clear that amino acids can also be selectively concentrated by some cells. It would be of value to have more data on the comparative distribution of such systems. The importance of such systems in metabolism seems clear, but details of this role are not available.

We have seen above that it is a primary necessity for a cell to possess an active ion transport system. We are entitled to expect therefore that such systems will be of general occurrence and also that variations on, and specializations of, such systems will abound. This is clearly a fruitful field for future investigation.

C. Composition of the Blood

In the higher animals the blood circulates in a system of closed channels, but in some lower animals (sponges, coelenterates, nematodes, rotifers, most arthropods, and many mollusks) an open circulation is present [for further discussion see Chapter 15 in Prosser (*35*)]. The immediate environmental fluid of tissue cells (and the interstitial fluid) is thus derived in one or more steps from the blood. Table III collects some of the data on the blood composition of various species. [For further data see Prosser (*35*), also Robertson (*62*), Bone (*158*), Roeder (*159*), and Florkin (*160*).]

Two well founded generalizations emerge from these data: (*a*) The ionic ratios are in many cases similar to those of sea water (*166*). (*b*)

TABLE III
Ionic Composition of Body Fluids

Animal	References	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}
Sea water (approx.)	*35*	440	9.5	10	50	520	30
Coelenterates							
Aurelia	*35*	429	12.4	9.2	43.7	485	22.1
Cyanea	*35*	420	14.9	9.6	50.2	540	10.5
Echinoderms							
Echinus	*35*	530	13.3	12.8	50.3	549	
Asterias	*35*	460	8.3	9.0	30.7	505	25.6
Annelids							
Amphitrite	*35*	406	13.0	9.5	54.8	477	30.8
Echiurus	*35*	439	12.6	9.2	42.5	480	30.8
Earthworm *Pheretima*	*35*	41.3	18.8	4.25	2.88	14.3	0.34
Mollusks							
Eledone	*35*	425	12.2	11.6	57.2	480	43.1
Mytilus	*161*	502	12.7	12.6	56	589	30.8
Anodonta (freshwater)	*161*	15.6	0.49	8.4	0.19	11.7	0.76
Arthropods							
Carcinus	*35*	513	11.9	12.9	19.0	540	20.0
Cancer	*35*	459	10.2	11.9	21.9	479	18.8
Homarus	*35*	455	9.28	17.1	9.0	472	5.1
Ligia (isopod)	*162*	586	14	72	42	600	9
Palaemon (prawn)	*163*	394	7.7	25.2	25.2	430	5.2
Astacus (freshwater)	*35*	152	2.8	12.0	2.48	176	
Insects (terrestrial)							
Hydrophilus	*35*	119	13.3	1.1	20	40	0.14
Dixippus	*164*	11	18	7	108	87	
Sialis	*165*	109	5	7.5	19	31	
Sphynx	*35*	4.35	48.4	7.48			
Apis (honey bee)	*35*	5.0	24.2	3.73	7.81	33.2	
Fish							
Myxine (hagfish)	*35*	558	9.6	12.5	38.8	592	61.1
Lampetra (lamprey)	*35*	119.6	3.21	3.93	4.21	95.9	5.24
Muraena (conger)	*35*	212	1.95	7.73	4.85	188.4	11.35
Lophius	*35*	228	6.4	2.3	3.7	164	
Amphibia							
Frog	*35*	103.8	2.5	2.0	1.2	74.3	
Mammals							
Rat	*35*	145	6.2	3.1	1.6	116	
Man	*35*	145	5.1	2.5	1.2	103	

The further, in its evolutionary development, that a group of animals is from its original marine ancestor, the less does its blood resemble sea water.

In some lower marine organisms (for example, the sea urchin, *Echinus*), the body fluid is a simple dialyzate of sea water. In species possessing powers of ionic regulation a varying degree of control of blood composition is achieved by differential ion uptake and excretion, but even here the resultant ionic composition still closely resembles sea water, for example, the body fluid of coelenterates and echinoderms (which is not regulated ionically), and marine annelids, mollusks and marine arthropods (all with powers of regulation). On the other hand, freshwater mollusks and arthropods have lower ionic concentrations than their marine relatives, in adaptation to the environment. In fact the bivalve *Anodonta* has the lowest concentration recorded of any animal, equivalent to 3% sea water (*161*). The blood of the earthworm has also a low salinity. Some insects are atypical in having reversed sodium/potassium ratios, a high potassium, low sodium plasma (*158*, *167*). Duchâteau and associates (*168*) show an evolutionary sequence of insects ranging from the primitive high sodium hemolymph type (Odonates) via intermediate states to the high potassium hemolymph type (Lepidoptera, Coleoptera, and Chrysomelidae). It is suggested that the change is due to the adoption of an herbivorous diet. Among the vertebrates, the marine teleosts possess a plasma ionic concentration half that of their environment. Amphibia have still lower tonicities, while mammal plasma is, again, a little higher.

In spite, therefore, of changes in total ion concentration in adaptation to environmental influences, the ion *ratios* are tenaciously held at the primitive levels. There has been much discussion of the physiological significance of the data [see Heilbrunn (*169*), Höber (*5*), and Seifritz (*170*)]. It must be remembered, however, that there are exceptions to the general rule, for example, in the case of the herbivorous insects. Furthermore, as we have seen, the cell interior possesses in general very different ion concentrations from the extracellular fluid. Thus many "explanations" fail which view the observed ion ratios in terms of a general effect on protoplasmic activity because these views ignore the fact that both the observed ratio and its reciprocal are compatible with (and probably essential for) vital function. Appeals to the "toxic" action of a particular ion (cf. magnesium and potassium anesthesia) are likewise insufficient since they beg the question of why the ion is, in the first place, toxic. On the basis of the fixed anion hypothesis it can be suggested that the high external sodium, internal potassium situation is primary and a response to the high sodium content of the oceans. The adaptation of cells and organisms to this is a secondary phenomenon. It is to be expected that cases will be discovered of intracellular biochemical systems with a specific need for potassium and likewise extracellular sodium-requiring systems, but it is suggested that these need not be interpreted in terms of a general protoplasmic (or even colloidal) effect.

D. BALANCE ACROSS THE CAPILLARY MEMBRANE: THE INTERSTITIAL FLUID

The organization of cells into tissues introduces the possibility of molecular diffusion between cells as well as through the cells. This distinction is fundamental to the physiology of the capillary membrane [see Drinker and Yoffey (*171*), Pappenheimer (*172*), and Davson (*4*)].

In animals of open circulation (see above), the interstitial fluid is in direct communication with the circulating fluid. In higher animals the capillary membrane of the blood vessels forms a barrier between the two fluids. In the vertebrates the lymphatic system is further interposed between blood and interstitial fluid (*35*). The walls of the lymphatic system, however, present no barrier to diffusion, and the composition of lymph and interstitial fluid is assumed to be identical in a particular tissue [for a discussion of possible inadequacies of such an assumption see Manery (*119*).] The walls of the blood capillaries, on the other hand, are far less permeable to proteins, although still highly permeable to smaller molecules. Diffusion is largely between the cells (0.2% of the capillary surface) through pores of (in mammals) 30–45 Å (*172*) at a density of 1 to 2×10^9 pores per cubic cm. of capillary wall. There is no evidence for any active transport across the capillary membrane. The distribution of ions across the capillary membrane (as was originally pointed out by Starling) is determined primarily by the Donnan distribution imposed by the impermeable proteins. Some ion-binding (especially calcium) by proteins and organic acids (citrate) occurs and modifies the apparent Donnan distribution. The metabolism of cells in a tissue can be expected to modify the composition of the adjacent interstitial fluid to an unknown extent (Manery). Average values for the ion ratios experimentally determined for a number of mammals (collected by Manery) are as follows (X_s and X_f are concentrations in serum and extracellular fluid respectively):

$[Na]_f/[Na]_s = 0.91$ $[K]_f/[K]_s = 0.76$
$[Ca]_f/[Ca]_s = 0.79$ $[Mg]_f/[Mg]_s = 0.87$
$[Cl]_s/[Cl]_f = 0.98$ $[HCO_3]_s/[HCO_3]_f = 0.99$

The ratio will depend on the relative protein contents of blood plasma and interstitial fluid and hence will be different for other phyla.

E. COMPOSITION OF TISSUES AND ORGANS

There is a great deal of information available on the water, electrolyte, and, in a smaller number of cases, nonelectrolyte composition of tissues and organs of many animals and plants. In most cases the distribution of the component studied between intracellular fluid and extracellular fluid is not known. Much of the data on electrolytes reflect simply the varying proportion of intracellular fluid to extracellular fluid in the different tissues. The reader is referred to the compilation of Manery (*119*) for data on mammals [also Harris (*124*), Prosser (*35*), and Heilbrunn (*169*)]. Thus, connective tissue, being mainly an extracellular phase, is, in general, rich in sodium and chloride. The eye lens, on the other hand, is low in chloride and high in potassium (121 meq./liter) and, correspondingly, contains a high proportion of intracellular matter. Bone, cartilage, and salivary glands have ion compositions similar to that of a plasma ultrafiltrate. In liver and kidney, chloride and sodium are both low, these tissues being highly cellular.

The mechanisms determining the balance of extracellular and intracellular components in complex tissues such as kidney are beginning to be elucidated. Thus, for example, Deyrup (*172a*) studying the behavior of slices of rat renal cortex incubated in solutions of varying ionic concentrations, in the presence of oxygen and glucose, finds a striking capacity for salt and water regulation in these circumstances.

F. ABSORPTION AND SECRETION

Tissues and organs concerned with absorption and excretion demonstrate the membrane control properties of their constituent cells specialized to a high degree. In many cases, the component transported has been demonstrated to move against an electrochemical gradient [sodium across the frog skin (*86*), glucose across the intestinal wall under defined experimental conditions], but this is clearly not a necessary feature of absorption and especially so of secretion, for example, the initial stages of glucose absorption after a heavy meal of glucose (*14*), secretion of enzymes in the pancreas (*173*). In absorption, metabolic incorporation of the transported component, and in secretion, metabolic synthesis determines the concentration gradient so that active transport need be of no consequence.

Absorption and secretion of a great variety of molecules and ions has been reported: water [Kitching (*38*), Visscher (*174*)], anions (*121*, *174*), cations (*175*, *176*), amino acids (*177*), sugars (*178*, *179*), proteins (*180*). (For reviews on absorption see Chapter 34 in Hober (*5*); Chapter 12 in Davson (*4*); Chapter 18 in Heilbrunn (*169*). For secretion see Chapters 35, 37 in Hober (*5*); Chapter 13 in Davson (*4*); Chapter 22 in Heilbrunn (*169*), the latter for comprehensive references to the early literature. For a comparative treatment of feeding and digestive mechanisms, see Chapter 6 in Prosser (*35*). The field is so vast that it is not possible to give here more than an eclectic treatment and the reader is referred to the previously cited treatises. Although in all cases absorption or secretion will affect the balance of the component concerned (or a derivative of it in the metabolic chain), we shall only be concerned here with those phenomena where the aspect of balance is of prime interest.

Absorption and secretion in unicellular organisms has been discussed in Section III,B. It is of interest that absorption of potassium by the yeast cell is linked to a secretion of hydrogen ions. The relation, which may be of most general significance, between this phenomenon and the secretion of hydrogen ions by the gastric mucosa in vertebrates is pointed out by Conway. Protozoa especially but also many other marine organisms secrete calcareous shells, mantles, and similar coverings. That such processes may be related to the maintenance of particular levels of calcium (and carbonate and phosphate) in the body fluid is suggested by the work of Potts on the mussels *Anodonta* and *Mytilus*. In a number of animals studied, the solubility product of calcium and carbonate ions exceeded that of aragonite, one form of crystalline calcium carbonate. Deposition (i.e., secretion) of calcareous deposits can be predicted under such circumstances (provided crystallization nuclei are available) and thus on the one hand a definite limit is set to the free calcium and carbonate in the body fluids while on the other hand a method is available (metabolic production of carbon dioxide) for control of calcium levels. Similarly, in the female stick insect most of the ingested calcium leaves the body in the eggs (*181*).

Ionic regulation in even simple marine animals involves the secretion of calcium and magnesium (see above). Life in fresh water requires the development of salt-absorbing systems in both the kidney and extrarenally (*49, 69*); the return of fishes to a marine environment is associated with the development of salt-excreting systems in the gills and in the kidney. Details of the kidney function in ion regulation have been most successfully elucidated in insects (*181*) and in the frog (*182*) and mammal [reviewed by Mudge (*183*)]. Using elegant microanalytical techniques on isolated Malpighian tubules of Dixippus, Ramsay was able to investigate ion movements in the insect kidney. Potassium was actively transported from the hemolymph into the Malpighian tubule. Water followed osmotically the movement of this potassium secretion, and the ions and other diffusible constituents were carried in by bulk flow (but both water and sodium could be actively transported, the latter under abnormal experimental conditions). The contents of the Malpighian tubule entered into the intestine—potassium, sodium, and water being reabsorbed in the rectum. Even under a high potassium loading there is such reabsorption of potassium, and it is suggested that this circulation of potassium is necessary for the continuous formation of the Malpighian fluid and concomitant excretion of nitrogenous waste. Treherne (*184*), using isotopes, has confirmed that the anal papillae of these larvae actively take up sodium.

Ramsay has now extended the study of insect kidney function by considering the fate of organic substances: amino acids, sugars, and urea (*69a*). These appear to be carried into the Malpighian tubules by simple diffusion and are then reabsorbed from the urine by the rectal glands. The analogy with the glomerular kidney is thus quite close. "The filtration and reabsorption process, available to vertebrates by virtue of their closed blood system and high arterial pressure, is replaced by a diffusion-reabsorption process," the bulk diffusion following the active secretion of potassium ions.

The situation in the insect kidney is thus interestingly different from those cases where the fluid in the excretory tubule is formed by filtration under pressure, for example, across the capillary membrane of the glomerulus in vertebrates. Subsequent treatment of the filtrate is in general similar to that in insects—there is reabsorption of essential ions, water, and also amino acid and glucose. Direct secretion into the tubule (the analogy of the process in insects) has been shown for a number of substances foreign to the organism, for example, Diodrast, *p*-aminohippuric acid, and also, significantly, potassium (*183*). Thus in *Necturus* kidney, potassium virtually disappears from the urine in the proximal tubule to reappear in samples taken from the distal segment (*185*). The thiosulfate ion is also reabsorbed and secreted. The function of vertebrate kidney in ion and water regulation is being investigated *in vitro* using slices of kidney cortex. These preparations accumulate potassium in exchange for sodium under conditions in which metabolism can occur [reviewed by Mudge (*183*)] reminiscent of the result of intracellular fixed anions. Investigations at an even more fundamental level show that the intracellular inclusions demonstrate such ion-accumulating properties (see later).

Absorption and secretion across the intestinal wall has been mainly investigated in mammals. Ions (*176*), glucose, and amino acids can all be actively transported under experimental conditions but it is calculated that 35% of glucose absorption under physiological conditions is not against an electrochemical gradient (*186*). Recent work is against the view that sugars are phosphorylated during their active transport, the specificity of the sugar-absorbing systems being dissimilar to that of sugar phosphorylases (*15*, *16*, *179*). Also, 3-methylglucose, which is almost certainly not phosphorylated *in vivo*, is actively absorbed by the cat intestine (*187*). Cordier and Maurice (*188*) have studied the absorption of sugars by the fish intestine.

In the opposite direction, the secretion of acid by the gastric mucosa of mammals has intrigued numerous investigators (*189–191*).

The parietal cells of the mucosa secrete a fluid which is (at its maximum concentration) isotonic hydrochloric acid corresponding to a pH of 0.85 (*192*). The acid must be formed at the cell wall since studies with indicators show the cell cytoplasm to be at approximately neutral pH (*190*). Formation of acid is accompanied by the production of equivalent amounts of bicarbonate ions in the cytoplasm which then exchange with plasma chloride ions. The basis of acid production thus appears to be the separation of hydrogen ions from bicarbonate at the parietal cell membrane. The tissue is rich in carbonic anhydrase, an enzyme which can form the required carbonic acid from carbon dioxide and water. Stimulation of acid secretion can be brought about by, for instance, histamine, and the extra acid so formed has been compared to the increase in oxygen uptake by the tissue on histamine stimulation (*190*). For each mole of oxygen consumed, 12 to 13 moles of acid are formed. Calculations show that 1 mole of oxygen oxidizing carbohydrate would produce just sufficient energy to secrete this amount of acid against the prevailing electrochemical gradient. The molecular mechanism by which the separation of hydrogen and bicarbonate ions occurs is still not established, but it is suggested that changes in the valence state of the iron in cytochrome is involved (*190*).

The cerebrospinal fluid and aqueous humor in mammals has, until recently, been considered to be a plasma ultrafiltrate. However, recent careful investigations by Davson and his co-workers (*193*) have required a modification of this view. Thus, for example, dialysis of aqueous humor against plasma *from the same animal* shows a small but definite migration of both sodium and chloride from the humor to the plasma, the ratio of Na^+ (plasma) to Na^+ (humor) rising from 1.04 before dialysis to 1.06 after, that of chloride from 0.945 to 0.97. Both the cerebrospinal fluid and the aqueous humor must now be regarded as formed by a secretory process. In support of this view, Nixon (*194*) has found that in both the cerebrospinal fluid and the aqueous humor, intravenously injected inositol is present at a higher concentration than in the plasma and is presumably secreted into the two former fluids.

In the cephalopods analogous ocular fluids are present. Robertson (*195*) has compared analyses of the ionic components of ocular fluids with plasma in a number of cephalopods. The two fluids differed in every component, the vitreous humor (liquid in these animals) being especially low in magnesium. A process of secretion is thus postulated here too. The aqueous humor in *Sepia* is of interest. This fluid is in communication with the sea water of the environment through a small pore in the cornea. Yet the composition of the fluid is neither that of sea water, nor of a dialyzate, nor again of a mixture of sea water with vitreous humor and it is considered to be a specific secretion.

Data exist for many other secretions but the organs concerned are often of only limited distribution among the phyla, e.g., sweat glands, (*196*) and cannot be discussed comparatively (see references quoted above).

G. Distribution between the Cytoplasm and Intracellular Inclusions

Information on the distribution of diffusible substances *within* the cell has been obtained using the new methods of isolation of cell components by differential ultracentrifugation (*197*) and analysis by flame photometry. The data so far refer only to vertebrate tissues—except for enzyme localization (*198*) though there is no reason why this should remain the case in the future. Protozoologists especially should find such studies rewarding since the phenomena of, for example, contraction and conduction displayed by unicellular organisms suggest some intracellular differentiation and localization.

The work on ion distribution in mitochondria (mammalian liver and kidney) may be found in the papers of Bartley and Davies (*199*), and is reviewed by Harris. Briefly, isolated mitochondria swell if metabolism is interrupted and their water content increases. At the same time, their powers of ion accumulation are lost (recalling the fixed anion effect in whole cells) but the effect of metabolism is supposed to be an indirect one, controlling the level of free calcium which then determines the internal water content. At very low external sodium concentrations ($6 \times 10^{-4} M$) a 26-fold concentration of this ion is achieved. Other gradients found are as in Table IV (from Davies, *200*). The mitochondria demonstrated a very high turnover of ions (probably by a rapid nonactive exchange) so that static ion-binding is not involved in the ion accumulation. In fact, the permeability of the mitochondrial membrane is similar to that of typical cells (*201*).

Data on ion distribution in nuclei have been obtained by Itoh and Schwartz (*202*). Sucrose-isolated nuclei had 15 meq. sodium and 42 meq. potassium per liter nuclei, while nuclei from nonaqueous media had 40 meq. sodium and 112 meq. potassium per liter nuclei. The membrane of the nucleus is considered by some workers (*203*) to contain pores of 300–600 Å but this may be an artifact (*28*). There is evidence that the membrane is permeable to proteins (*204*). A free ion permeability may perhaps therefore be expected.

Further evidence for the semipermeable nature of the nuclear membrane has appeared. Thus Battin has isolated amphibian egg nuclei (*Triturus*) and finds that these are permeable to sucrose and impermeable to serum albumen (*204a*). An elegant microinjection procedure has been used by Harding and Feldherr (*204b*), to study the osmotic properties of nuclei of intact cells, again amphibian oocytes (*Rana*). Molecules of molecular weight 40,000 and larger did not freely penetrate the cell nucleus.

TABLE IV

RATIOS OF INTERNAL/EXTERNAL CONCENTRATIONS IN MITOCHONDRIA FROM METABOLIZING SHEEP KIDNEY CORTEX AT 20°

Substance	Ratio	Concentration in medium after separation in moles per liter
"H^+"	2.5	1.6×10^{-7}
Na^+	1.5	2.6×10^{-2}
K^+	2.4	9.0×10^{-2}
Orthophosphate	6.0	1.9×10^{-4}
Fumarate	8.0	2.8×10^{-4}
Pyruvate	1.1	3.5×10^{-3}
Citrate	0.8	1.4×10^{-2}
Oxaloacetate	0.1	1.8×10^{-3}

Unfortunately, data for a complete balance sheet of diffusible components within the cytoplasm are not yet available, although a start in this direction has been made (*205*). The cell organelles in the cytoplasm have presumably similar problems of hydrodynamic equilibrium as have cells in interstitial fluid, but data for a similar analysis are lacking.

IV. Balance in Plants

A. PROBLEMS OF BALANCE IN PLANTS AND ANIMALS COMPARED

Plants differ so much from animals in their morphology that quite different problems are posed in the analysis of balance in plant materials. The general absence in plants of internal organs and systems composed of discrete phases separated by membranes (e.g., in animals the blood and interstitial fluid separated by the capillary walls) removes the necessity for consideration of such transmembrane balance problems within the plant. Indeed, some workers consider that all the protoplasm in a plant is in direct syncytial communication, i.e., is one phase—the symplast. (We neglect here the probable phase barrier between the cytoplasm and the intracellular organelles.) Balance between the plant, considered as one phase, and the environment has been much studied but, since most work has been done on terrestrial angiosperms (flowering plants), generalizations of a comparative nature (cf. MacAllum's hypothesis (*166*) concerning ionic balance in marine animals) have not arisen. Most work has been concerned with the *mechanism* of water, electrolyte, and nonelectrolyte uptake and internal distribution and not with the comparative aspects of balance. In what follows, work relevant to this present theme of balance will be discussed, first in the unicellular plants and in single cells of higher plants (where the conditions are closer to those in animals). Subsequently the water and solute relations in more complex plant material will be considered.

B. Balance in Single Plant Cells

In mature plant cells, the major portion of the interior of the cell is filled not with protoplasm but with a largely protein-free aqueous solution of ions and small organic molecules, the vacuolar sap. The protoplasm exists as a thin (of the order of 1μ) layer outside the vacuole but internal to the cellulose wall of the cell. Thus a solute passing from the exterior to the vacuole has to cross successively the cellulose cell wall, the exterior surface of the protoplasm (known as the plasmalemma), the protoplasmic layer itself, and finally the inner boundary of the protoplasm (the tonoplast) before entering the vacuole (*206*). The major barrier to diffusion of solutes is presumed to be at the tonoplast membrane (but see below) (*207*). The protoplasm (a fairly rigid gel) is considered by Briggs to form part of the "outer space" or "apparent free space" into which diffusion of externally applied solutes, e.g., electrolytes and mannitol, is not restricted (*208*). If this view of the ready permeability of the plasmalemma is valid, then balance of electrolytes across the plasmalemma must be determined by the Donnan potential due to the protein in the protoplasmic gel together with possible specific binding by cytoplasmic constituents. Unequivocal evidence for this hypothesis is not yet available (*207*). Estimates of the concentration of the fixed (Donnan) anions in the protoplasm range from 20 meq./liter in carrot tissue to 830 meq./liter for beet (*208*). [In animal tissues (see Section III,B) the concentration of fixed anion is of the order of 35 meq./liter in the ox red cell, increasing to 420 meq./liter in nerve cells.]

The high permeability of the plasmalemma interface requires that the mechanisms responsible for active maintainance of any concentration gradients that may exist between the vacuolar sap and the external solution be situated at the tonoplast, if economic maintenance is to be achieved (cf. Section I,B). Direct evidence for the existence of such concentration gradients comes from studies on the very large coenocytic algae, e.g., species of *Valonia, Nitella,* and *Halicystis,* introduced as experimental material and intensively studied by Osterhout (*209*) and Hoagland (*210*). The coenocyte of *Valonia,* indeed, may be as large as a pigeon's egg and specimens of sap can readily be withdrawn and analyzed. Some of the data available are collected in Table I.

Osmotic and electrical conductivity measurement show that the thermodynamic activity coefficients of the ions in the vacuolar sap are not far removed from those in the external medium, i.e., there is little static ion binding by vacuolar constituents. Measurements of the electrical potential developed between the inside and outside of these large cells have been made, with interesting results (*5, 209–212*). That the membrane potential has a complex basis is shown by the experiments of Blinks (*211*) who found that if, in *Halicystis,* the vacuolar sap is replaced by sea water (the external medium), the membrane potential remains unchanged at +80 mv. (outside positive) and is thus an asymmetry potential. The total potential developed between the inside and outside of the cell is the algebraic sum of the potential across the plasmalemma (estimated for *Halicystis* by Blinks as +120 mv.) and that across the tonoplast (estimated at —40 mv.). There are indications that the hypothesis of a freely permeable plasmalemma does not apply to these large cells. Thus Walker has shown by direct measurement that the major part of the electrical resistance between the inside and outside of the cell of *Nitella* resides at the plasmalemma (*212*). Also the calcium ion is not in Donnan equilibrium between the cytoplasm and environment. However, experiments on the uptake of rubidium and radioactive potassium by *Valonia* and *Nitella* (see reference *213*) show that these ions are accumulated rapidly initially in the cytoplasm (rubidium to some fortyfold the external concentration). Only secondarily are they transferred into the sap (where they are finally at a higher concentration than in the external medium). Thus the main barrier to diffusion, using this technique, would seem to reside at the tonoplast membrane.

However, in general, balance in single plant cells can probably be considered as being determined by an initial passive accumulation in the cytoplasm, the distribution of ions being influenced by the prevailing Donnan potential due to the fixed anions in the cytoplasm. Subsequently simple diffusion, but also active transport across the internal tonoplast membrane may occur, so that the final concentration of ions and neutral molecules in the vacuolar sap can be very different from that in either the cytoplasmic border or the external medium.

Recent work of MacRobbie and Dainty on the alga *Nitellopsis* (*157c*) requires some modification of the above view on ion balance in single plant cells. Careful determinations, using isotopic tracers, of the movements of potassium, sodium, and chloride ions between the cell and the environment require kinetic explanation in terms of a model, postulating three major compartments in the cell. These are: (*a*) the outermost free space of the cell, comprising probably the cell wall; (*b*) the "protoplasmic nonfree space," perhaps to be identified with the protoplasmic lining of the cell—or some fraction of this; and (*c*) a slowly exchanging compartment, fairly identified with the cell sap. The data suggest that both sodium and chloride are actively transported between the cell sap and the exterior, the potassium moving passively. This three-compartment model is confirmed for potassium and the brackish-water alga *Nitella* by the work of Diamond and Solomon (*213a*). Both the tonoplast and the plasmalemma are here found to be significant and commensurate barriers to potassium diffusion. MacRobbie and Dainty present good evidence that a sodium-potassium exchange mechanism is situated at the outer (plasmalemma) membrane, which membrane appears to be analogous with typical animal cell membranes. The inner (tonoplast) membrane is very different, possessing a mechanism for the secretion of chloride into the vacuole (and may perhaps be fruitfully compared with the chloride secretory system of the mammalian gut). Work on the nonvacuolated alga *Porphyra* [Eppley (*157b*)] confirms that a plant cell can actively extrude sodium and accumulate potassium in a manner reminiscent of animal cell membranes.

C. Balance of Ions and Water in Plants

In spite of the powers of selective ion accumulation possessed by the living plant (see below), resulting in a potential ability to regulate the ratio of internal and external concentration of ions, the plant is by no means independent of the concentrations of the bulk ions in the environment. [Inability to regulate the concentration of the trace elements gives rise to much-investigated toxic and deficiency diseases (*214, 215*).] In part, the effect of bulk ions is a physical one as is that of the colloidal structure of soil. Thus, the "alkali" soils of the southwestern United States are unfavorable for plant growth, the high sodium ion concentration producing a high degree of dispersion of the soil colloids and thus affecting the water-retaining properties of the soil (*216*). But even plants grown in aqueous salt solutions ("soil-less culture") are sensitive to the ionic ratios in the environment. Osterhout (*209*), inspired by the early work of Loeb on animal cells, was able to demonstrate marked antagonism between the monovalent and the divalent and trivalent cations in the alga *Laminaria* grown in artificial salt solutions. Electrical conductivity measurements showed the resistance of the thallus of this alga to vary with the relative concentrations of ions in the salt solutions. Monovalent cations decreased the electrical resistance, di- and trivalent cations having the opposite effect. The results were interpreted in terms of effects of the ions on the degree of hydration of the outer protoplasmic layer. Thus, on the Briggs-Robertson interpretation (see above), in spite of the cell possessing the ability to accumulate ions selectively in the vacuole, ions in the immediate environment can affect the vitality of the cell by a direct (and possibly largely uncontrolled) effect on the protoplasm of the "apparent free space." Table V records the composition of some of the aqueous solutions that have been found by different investigators to be optimal for many plants. It may be pointed out here that in contradistinction to animals, plants do not seem to require appreciable quantities of sodium.

TABLE V

BALANCED SALT SOLUTIONS FOR OPTIMAL PLANT GROWTH[a,b]

Due to	K^+	Ca^{2+}	Mg^{2+}	NO_3^-	PO_4^{3-}	SO_4^{2-}	Cl^-
Knop (1860)	350	340	80	880	150	80	—
Pfeffer (1900)	620	490	170	1180	150	170	270
Shive (1915)	180	50	150	100	180	150	—
Hoagland (1920)	60	50	20	60	10	20	—

[a] Calculated from compilation of Curtis and Clark (*217*) in meq./liter.

[b] In all cases, traces of iron as the chloride, phosphate, or tartrate are added.

The concentration of ions available to the plant in the soil water can be estimated by leaching of soil samples (*216*, *218*). Both the methods of determination and the concept of available ions must be considered with due caution. A typical analysis (according to Hoagland) is shown in the tabulation.

Cations (meq./liter)		Anions (meq./liter)	
K^+	0.62	NO_3^-	2.8
Na^+	2.1	PO_4^{3-}	0.017
Ca^{2+}	7.1		
Mg^{2+}	8.2		

This may be compared on the one hand with the data of suitable aqueous salt solution growth media in Table V and, on the other hand, with the data shown in Table VI, in which the concentration of certain

TABLE VI

CONCENTRATION OF ELEMENTS[a] IN GREEN TOPS OF DIFFERENT PLANTS GROWN IN THE SAME SOIL SAMPLE (LOAM)

Plant	K^+	Ca^{2+}	Mg^{2+}	N	P
Sunflower	125	59	42	147	36
Bean	52	62	40	180	29
Wheat	22	24	19	330	38
Barley	20	33	23	270	78

[a] Calculated from Newton (*219*) mg. atoms/kg. fresh tissue.

elements (in the green tops) of various young plants is given, the plants being grown under closely similar soil conditions in the greenhouse. These data are a clear reflection of the differing capacities for ion regulation demonstrated by these plants. That these powers of ion regulation are not unlimited is clearly shown by the data in Table VII which

TABLE VII

REGULATION OF INTERNAL OSMOTIC CONCENTRATION IN A PLANT (MAIZE)[a]

Osmotic pressure of growth medium (in atmospheres)	Osmotic pressure of sap from root cells (in atmospheres)
1.21	4.59
1.99	5.48
3.38	6.61
4.96	7.51
7.22	8.19

[a] Data of McCool and Millar (*220*).

presents data on the osmotic concentrations for a set of plants grown in solutions of different concentration. Over a 6-fold range of external salt concentration, the concentration of salt rose by less than 2-fold, showing that ion regulation was possible, but only to a limited extent. However, plants grown in culture solutions of low concentration, continuously maintained, make almost as good a growth as those of high concentration likewise maintained.

In soils of a high salt concentration, resulting osmotic effects begin to be of importance (*221*). As will be discussed later, water is presumed to enter terrestrial plants down an osmotic gradient. This gradient is reduced if the soil salt concentration is increased. The data in Table VII may be looked upon as in part an adaptation of the plant in response to the high salt ratio in the environment. Species of plants growing in extremely salty environments, e.g., in salt marshes, are found to have exceedingly high internal osmotic pressures (e.g., over 200 atm. for saltbush *Atriplex confertifolia*, compared with 7 to 10 atm. for the commoner plants). Such plants can grow readily in ordinary soil however (*221*).

A terrestrial plant loses a relatively enormous amount of water by *transpiration*—the direct evaporation of water from the surface of the plant. As much as several times its own volume of water may be lost by a plant on a hot dry windy day. To achieve balance, this water loss is made up by absorption of soil water by the roots. The water loss by transpiration is a direct consequence of the necessity for gaseous exchange at the leaf surface and is considered by some authors not only to lack a physiological function but, indeed, to be a "necessary evil" (*217*). While absorption of water is largely a result of water loss by transpiration (see below) and can never (except during recovery from dehydration) exceed transpiration, a water deficit due to excessive transpiration is of common occurrence and, indeed, occurs daily in many plants in temperate zone summers (*216*). A water deficit can result either from an increased rate of transpiration or decreased absorption. Wilting is a common symptom of water deficit; as the water deficit increases, a plant undergoes transient (or reversible) wilting, permanent (irreversible) wilting, and eventually death (*222*). A high transpiration rate is favored by atmospheric conditions such as heat, dry air, and wind, all of which produce a local decrease in humidity at the leaf surface. Many species of plants have developed specialized leaf structures which reduce transpiration, in adaptation to life in drier climates.

In plants generally, gaseous exchange and hence water loss by transpiration is confined to a small area of the leaf surface and occurs via minute pores, the stomata. The remainder of the leaf is covered by a relatively impermeable layer, the cuticle. In plants living in a dry climate, the stomata are often sunk below the level of the epidermis, raising the local humidity and reducing water loss. The stomata may be confined to the under side of the leaf where a cooler and milder microclimate prevails. In extreme cases, stomatal opening and hence gaseous exchange may occur only at night, reducing transpiration to low levels. Another adaptation is a further increase in the impermeability of the cuticle. Cuticular transpiration is reduced from about 10% of the total transpiration loss in common plants to less than 1% in drought-resistant species (*222*).

The rate of absorption of water from a given soil depends on the physiological state of the plant; a nontranspiring plant in a swollen, turgid condition will not absorb water, while a plant which has been dehydrated to wilting will rapidly absorb if water is available. The barrier to rapid absorption is believed to reside in the resistance to water movement offered by the cortex of the root. Intact plants usually require over half an hour to recover from wilting. If the roots are removed and the plants again immersed in water the leaves recover turgidity in 5 minutes (*216*). Soils differ in the amount of their constituent water that can be made available to the plant, the tenacity with which water is held by the soil depending largely on its colloidal structure. A physiological measure of this tenacity is the "permanent-wilting percentage" (*223*) which is the moisture-content of the soil which just supports growth of the plant. (Below this moisture-content, the plant can still absorb water from the soil but not at a sufficient rate to offset the transpiration loss.) Table VIII records values of the "permanent-wilting percentage" for different soils and for different plants in these different soils. The surprising conclusion (still considered valid) (*222*) reached by Briggs and Shantz on the basis of these data was that there was no important difference between different species of plants in their ability to reduce the moisture content of the soil before wilting. That is, a plant adapted to arid conditions cannot extract a greater percentage of water from a given volume of soil than can a plant adapted to a moist climate. Plants which thrive on dry soils have highly developed root systems, enabling them to extract the available water from a larger volume of soil than is usual in other plants. Apart from drastically reducing transpiration loss (see above), the major factor allowing drought-resistance in plants is the ability to survive long periods of dehydration which would kill more common species. A similar explanation has been put forward to explain the ability of the camel to withstand desert conditions (see Section II,C,8,*f*).

TABLE VIII

PERMANENT WILTING PERCENTAGES OF VARIOUS SOILS MEASURED USING DIFFERENT PLANT SPECIES[a]

Species	Coarse sand	Fine sand	Sandy loam	Loam	Clay loam
Corn	1.07	3.1	6.5	9.9	15.5
Sorghum	0.94	3.6	5.9	10.0	14.1
Wheat	0.88	3.3	6.3	10.3	14.5
Oats	1.07	3.5	5.9	11.1	14.8
Peas	1.02	3.3	6.9	12.4	16.6
Tomato	1.11	3.3	6.9	11.7	15.3
Rice	0.96	2.7	5.6	10.1	13.0

[a] Data of Briggs and Shantz (*223*).

D. Osmotic Problems in the Absorption and Translocation of Water and Solutes

Water and mineral ions are absorbed by the roots of the plant and travel (mainly) up the xylem (or wood) to the leaves and other sites of utilization. Metabolites, synthesized in the leaves, travel down to the roots for the most part in the phloem (or bark) (*210, 222*). In the course of these movements water and solutes have to pass a number of barriers, across which problems of balance may arise.

In particular; (*a*) entry into the root system occurs across the barrier presented by the cell membranes of the root epidermis; (*b*) movement from the cortex of the root into the interior occurs across the continuous layer of cells of the endodermis rather than between them, owing to structural modification of this layer (*222*); (*c*) thereafter, movement into the (dead) conducting elements of the xylem occurs across the cell membranes of the surrounding living cells, and barriers are present in the reverse direction during the passage of materials from the xylem to the sites of utilization of the materials.

Entry of water into the cellular components of the root occurs in response to the *diffusion pressure deficit* of the cell—that is, the difference between the osmotic pressure of the vacuolar contents and the turgor pressure exerted by the cell wall. Opposing these forces are the surface forces binding water to the colloid particles of the soil. The lateral movement of water across the root and into the xylem can occur as a result of the difference in osmotic pressure between the soil water and xylem sap in spite of the presence of high osmotic concentrations in the intervening cells. Thus, if transpiration is reduced by cutting off the upper stem and the leaves of the plant, exudation of sap from the cut stem occurs and can occur against a pressure of a few atmospheres, a phenomenon known as *root pressure* (*224*). It is considered that, under these conditions, the cells between the epidermis and the xylem become fully turgid and, since the cells can no longer absorb water, water will move from the soil to the sap down the osmotic gradient, which is maintained by the secretion of salts into the xylem (*210*). When transpiration is taking place, however, the water in the xylem is being continually removed and a gradient of diffusion pressure deficit is thought to be maintained across the cells of the cortex and the region surrounding the xylem. The mechanism of the movement of water up the xylem is controversial (*225*) but in the last analysis, energy for this process comes from the radiant energy absorbed during the evaporation of water in transpiration. Only in the development of root pressure (of generally minor physiological importance, but perhaps more so in the spring, when the rate of transpiration is low) is metabolic energy required for water movement in the plant. Even here, the movement of water is passive in the sense that it occurs down the osmotic gradient. That water can move against an osmotic gradient has been suggested by various workers but the evidence for this is not conclusive (*226*).

A comparison of the data of Tables V and VI might suggest that the uptake of salts is necessarily active since the ions in the plant are, in general, at a higher concentration than in the soil water. However, the possibility exists that this accumulation may be due to concentration of the soil water as a result of evaporation during transpiration. In fact, the low ion permeability of the cell membrane forbids this interpretation, assuming as it does a mass flow of the soil solution into the plant. The active uptake of ions by plant roots is indeed demonstrable. Thus Lundegårdh collected the sap exuded from the cut ends of roots immersed in 1 m*M* KNO_3 and found the sap to consist largely of an 18 m*M* solution of this salt (*227*). No transpiration could, of course, occur under these conditions.

The mechanisms by which the active accumulation of ions is driven by metabolic processes are still obscure. Jenny and Overstreet (*228*) present evidence for the role of an ion exchange mechanism in the initial stages of ion absorption by which cations are taken up from the colloidal lattice of the soil in exchange for hydrogen ions. Lundegårdh (*229*), on the other hand, is of the opinion that it is the anions that are initially actively accumulated, the uptake of anions being correlated with a particular cyanide-sensitive fraction of the respiration of the root. A third school, that of Steward (*230*), stresses the particular role of actively growing cells in ion accumulation and seeks to link ion uptake with the disappearance of ion-binding centers during protein synthesis. It is probable that these viewpoints are not mutually exclusive (*231*). An authoritative and comprehensive review of salt uptake in plants has recently appeared [Sutcliffe (*231a*)].

The movement of ions from the root epidermis to the xylem is in the direction of decreasing aeration (*232*). Thus the cells surrounding the xylem may lose salts to the xylem sap by virtue of the resulting decrease in their ion-accumulating ability. A direct secretion of ions into the xylem has, however, been postulated by other workers. It is of interest that studies with radioactive bromide ions showed that salts, in their movement from the epidermis to the interior, could bypass the vacuoles in high-salt plants but, in plants depleted of salt, movement into the vacuoles as well as into the xylem occurred; that is, two pathways of salt movement exist.

The movement of metabolically synthesized sugars and organic acids from the leaves down to the lower regions of the plant occurs mainly in the phloem. There is no reason to suspect that movement of nonelectrolytes occurs against a concentration gradient although the mechanism of movement of the phloem itself is quite obscure (*233*).

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CHAPTER 9

The Mechanisms of Osmoregulation

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I. Introduction

Studies on the osmotic relations of animals commenced at the turn of the century with the classical investigations of L. Fredericq, Botazzi, and others on the composition of aquatic animals' body fluids, measured mainly by their freezing point depression. The results showed that whereas most marine invertebrates were in osmotic equilibrium with their environment, this was not generally true of the marine vertebrates or of freshwater animals. It was only a few years earlier that Pfeffer had found the relationship between the osmotic pressure of a solution and its concentration and Van't Hoff had introduced the term "semipermeable" for the membrane used to separate a solution from pure solvent in the measurement of osmotic pressure. These workers and other physical chemists were developing the osmotic theory of solutions at this time and it is hardly surprising that the concepts of osmotic pressure and the semipermeable membrane were taken over by the biologists in an attempt to account for the osmotic behavior of organisms and the properties of their bounding membranes.

The next 50 years saw, first, the demonstration of the permeability of the outer membranes of many marine invertebrates to the ions of sea water (*1, 2, 3*) and of the permeability of many others, including freshwater animals to sodium and chloride ions (*4, 5, 6*), so that today it seems probable that in the majority of animals the penetration of both water and salts is a common feature. The concept of the semipermeable membrane is clearly inadequate. The discovery of mechanisms located in the outer membranes of marine animals by which ions could be transported against a concentration gradient (*7, 8*) and their demonstration in many freshwater animals (*9*), together with the location of such processes in excretory tubules and in the alimentary canal, led to a gradual characterization of the processes by which osmotic pressure differences are maintained.

The more recent demonstration of the permeability of many cells to ions and the presence of specific transporting mechanisms located within the cell membranes themselves (*10, 11*) show that the bounding membrane of an animal may be simply exploiting a fundamental process possessed by the cells which compose it.

The osmotic behavior of animals and methods of osmotic regulation have been reviewed at intervals during the last forty years (*12–21*) and these reveal a gradual clarification of the mechanisms by which osmoregulation is achieved.

A steady state existing between an organism and its environment may be far from a true equilibrium. To maintain such a state, energy must be supplied to the system and this is done at the expense of the metabolic activity of the organism. In the absence of metabolism, or if the link between the energy source and the specific mechanism is broken, then the system will tend to revert to a simple equilibrium. In an organism many steady states may exist for many different types of molecules—osmotic pressure differences constitute one particular state where differences in chemical activity ("the effective concentration") of water molecules between the organism and its environment are maintained. The core of the problem of the maintenance of these differences is the means by which some of the energy, set free by metabolism, is utilized to achieve this end.

If the organism is bounded by a semipermeable membrane then the problem resolves itself, simply, into the means by which water is transported in a direction, and at such a rate, as to balance the movement of water due to osmosis. The demonstration of the permeability of animal membranes to ions has made the situation more complex. Since differences in osmotic pressure between the fluids of an organism and its environment are generally a result of differences of ionic concentration, the maintenance of osmotic differences involves the movement of both water and ions. There are two processes implicated: (*a*) the transport of water against the osmotic gradient, and (*b*) the transport of ions in a direction opposite to that of the net movement of ions due to diffusion. Both these processes require energy; but work is not necessarily done on the water molecules or the individual ions themselves. Transport may be achieved by indirect means.

The osmotic pressure of an organism may be lower than, equal to, or higher than that of its environment and these conditions may be referred to as the hypo-osmotic, isosmotic, and hyperosmotic states respectively. *Hyperosmotic regulation* is characterized by processes for the *uptake of ions* and the *expulsion of water. Hypo-osmotic regulation* requires the *absorption of water* and the *secretion of ions.*

The study of osmoregulation has been primarily concerned with the elucidation of the mechanisms by which these processes are achieved. Much less is known of the way these mechanisms are *regulated*, so as to maintain the constancy of the ionic composition and water content of the organism in the face of changes in diffusion rates. It is to this aspect of the subject that much of the future work on osmoregulation must be devoted.

It is important, at this stage, to make the distinction between osmoregulation in animals which possess a body fluid and osmoregulation in individual cells. In the first case, we are concerned with a steady state existing between the environment and body fluid—a system characterized by a bounding membrane which is divided into special regions, often with different permeability properties and usually differentiated into special organs, such as gills, gut, excretory system, and integument. The cell, on the other hand, is bounded by a membrane which is microscopically homogeneous and may separate the cell either directly from the external environment, as in the lower animals, or from the body fluid in the higher forms. Although the processes involved in osmoregulation of body fluids and cells are the same, the composition of cells and the nature of their membranes differ so much from the composition of body fluids and the outer membranes of the animal, that the mechanisms by which osmoregulation are achieved in the two cases must be considered separately.

We can, then, consider first the occurrence of anisosmotic states in animals with body fluids and the mechanisms by which these are achieved.

II. The Occurrence of Anisosmotic States in Animals

A. MARINE ANIMALS

The composition of the body fluids of many marine animals are known with a high degree of accuracy (*22–26*). Some of these analyses are shown in Table I where the freezing point depression and the concentration of the individual ions are expressed as a percentage of their values in sea water. The great majority of marine *invertebrates* are isosmotic with their environment (*13*) and no problems of osmoregulation are encountered; but there are a number of exceptions to this rule. The most important occur amongst the marine arthropods where some of the crabs, like *Pachygrapsus* (*27*), and the prawns, like *Palaemon serratus* (*28*), have an osmotic pressure distinctly below that of the sea water. Among the marine *vertebrates* the divergences are greater. Thus

TABLE I
THE COMPOSITION OF THE BODY FLUIDS OF SOME MARINE ANIMALS[a]

Animal	Freezing-point depression	Na	K	Ca	Mg	Cl	SO_4
Echinoderms							
Echinus esculentus (*22*)	100	100	102	101	100	100	101
Holothuria tubulosa (*24*)	100	101	103	102	104	100	100
Annelid							
Arenicola marina (*23*)	100	100	103	100	100	98	92
Mollusks							
Mytilus edulis (*24*)	100	100	135	100	100	101	98
Eledone cirrosa (*23*)	100	97	152	107	103	102	77
Arthropods							
Homarus vulgaris (*22*)	100	110	85	131	14	101	32
Carcinus maenas (*13, 26, 30*)	98–103	110	118	108	34	104	61
Pachygrapsus marmoratus (*24, 27*)	91	94	95	92	24	87	46
Palaemon serratus (*28, 31*)	74	79	70	115	22	74	9
Vertebrates							
Cyclostome							
Myxine glutinosa (*25, 29*)	102	110	90	56	33	97	22
Elasmobranchs (*29*):	102–110	—	—	—	—	—	—
Mustelus canis (*32*)	109	61	47	37	6	45	7
Teleosts (*29*)	34–44	—	—	—	—	—	—
Muraena helena (*25*)	—	38	16	31	4	29	17

[a] Values are expressed as a percentage of the corresponding values in sea water or in the body fluid dialyzed against sea water.

the hagfish, *Myxine,* is slightly hyperosmotic but the marine elasmobranchs are invariably significantly hyperosmotic and the marine teleosts maintain a blood concentration well below that of sea water (*29*).

An examination of the ionic composition of the body fluids of marine animals shows that some, like the echinoderms, *Echinus* and *Holothuria,* have a composition so close to that of sea water that the system must approximate to that of a true equilibrium. On the other hand, other isosmotic species show significant divergences in the concentrations of some of the major ions. Thus compared with sea water, *Arenicola* has less sulfate, *Mytilus* more potassium, and *Eledone* more potassium and calcium but less sulfate. In the higher crustacea, divergences in the concentrations of all the major ions may be found and magnesium and sulfate are often especially low. In *Carcinus,* for example, the permeability of the body surface to all the major ions of sea water is well established (*26*) so there can be no question of a simple equilibrium. The steady state must be maintained by the uptake of sodium, chloride, potassium, and calcium and the secretion of magnesium and sulfate, all against a concentration gradient. Until we have a knowledge of the electrochemical gradient (*11*) for each ion it is not possible to tell which ions are actively transported, but certainly some of them must be.

These and other similar studies (*14, 20, 26*) on marine invertebrates show us that mechanisms for the transport of ions (other than by diffusion) may be well developed in animals which display no powers of osmoregulation. Thus the movement of ions, fundamental to osmoregulatory processes needs no evolutionary innovations, but can result from the elaboration of already existing mechanisms. Reference to the hypo-osmotic forms, *Pachygrapsus* and *Palaemon* makes this point clear. As in *Carcinus,* there are divergences in the ionic ratios for all the major ions but the ion concentrations are, in these animals, all *lower* than in sea water and the lower *total* ion concentration is reflected in the lower osmotic pressure of their body fluids. This condition must be maintained by the outward transport of all ions and, also, by the uptake of water.

A comparable situation exists among the marine vertebrates. In *Myxine* which is nearly isosmotic, the observed ionic differences must be maintained by the secretion of all ions with the exception of sodium (Table I). In all the marine teleosts (*Muraena helena* serves as a typical example) every one of the major ions is secreted and low concentrations result. The low levels of sodium and chloride, especially, account for the low osmotic pressure of the blood, since these ions make up some 90% of the total osmotic pressure of sea water. As with all hypo-osmotic animals, the maintenance of the steady state in the teleosts also requires the uptake of water. The marine elasmobranchs constitute a special case which is unique in the animal kingdom. The concentration of ions in the blood is almost as low as in the marine teleosts yet the osmotic pressure is slightly greater than that of sea water (33). This is achieved by the retention of large amounts of urea in the blood by the reduction in permeability of the outer membranes to this substance and an effective reabsorption in the kidney tubules. Secretion of ions is still required to maintain the low concentrations found in the body fluid but the osmotic gradient is reversed and no water uptake is demanded. In this way a very considerable economy in the energy requirements for salt and water balance is effected.

Another feature which has important consequences on the development of osmoregulatory mechanisms is the possession of excretory organs by many of the higher animals. By means of these organs a continuous flow of water takes place through the animal, even in those forms which are completely isosmotic with their environment. In such animals, the water lost by excretion cannot be taken up by osmosis and must be absorbed by some other process. Thus, since mechanisms for the uptake and removal of water are present in many animals, the transport of water necessitated by an anisosmotic state may also be a simple extension of some process which already exists, as in the case with the inorganic ions.

The excretory organ also serves as one of the most important routes for the removal or secretion of ions. The fact that this organ is present in many isosmotic animals suggests that its function (apart from the removal of waste substances), together with the associated, and necessary, uptake of water and ions by some other part of the animal, is to regulate the volume of the body fluids.

A combination of the above conclusions makes it possible for us to visualize the evolution of osmoregulatory mechanisms as the elaboration of mechanisms whose primary function is the regulation of body volume. This could proceed in two stages. Firstly, the intensification of certain of the ion-transporting systems would lead to a regulation of the ionic composition of the organism. Secondly, a further development of ion transport and control of water movement would give the conditions necessary for the maintenance of osmotic pressure differences.

B. Brackish Water Animals

Bodies of water, such as estuaries and saline pools, where the salt content is lower than that of sea water but considerably in excess of that in fresh waters, support a varied but rather sparse fauna. The characteristic feature of these waters is the great variability in their composition, and this contrasts strikingly with the constancy of the marine environment. Many of the well known brackish water animals, like *Arenicola* and *Mytilus* (*13*), are isosmotic with the surrounding water at all concentrations in which they can survive. Animals such as these, are continually faced with problems of adjustment to their changing environment, but these problems are met with mechanisms for body volume control and by the adaptation of their cells: no osmoregulatory mechanisms are involved. Among the invertebrate fauna, powers of osmoregulation are restricted almost entirely to the arthropods—the polychaete, *Nereis diversicolor,* which is slightly hyperosmotic at concentrations between 20–50% sea water (*13*), is the only non-arthropod species where significant osmotic differences have been discovered. Among the crustacea osmoregulation is very well developed and representatives from several different orders show varying degrees of hyperosmotic control (*17*). The processes involved in the maintenance of the hyperosmotic state will differ slightly in different animals but may be illustrated in the case of *Carcinus,* living in dilute sea water. In 66% sea water the concentrations of sodium, potassium, calcium, and chloride are some 30–40% higher in the blood than in the water, whereas magnesium and sulfate concentrations are still distinctly lower (*26*). Therefore, in this crab, water, entering along the osmotic gradient, must be removed, together with magnesium and sulfate but all the other ions must be absorbed against the concentration gradient.

Perhaps the most interesting brackish water animals are those, like the prawn, *Palaemonetes varians* (*28*), certain insect larvae, like *Aedes detritus* (*34*), and some fish, especially migratory forms like the eel (*12*) which are all hypo-osmotic in sea water but, on dilution of the sea water, pass through a isosmotic stage, to become hyperosmotic in the more dilute solutions. These animals not only possess mechanisms for both hypo- and hyperosmotic regulation but must also exert a considerable control over them.

C. Freshwater Animals

The total concentration of the body fluids has been measured in many freshwater animals and is invariably greater than that of the surrounding water. A number of analyses of the blood or tissue fluid of freshwater forms are shown in Table II. A striking feature is the great range in concentration displayed by different species—for example, the blood of *Telphusa* is as much as 14 times as concentrated as that of *Anodonta*—and it is noteworthy that the vertebrates and the arthropods, which show the greatest powers of osmoregulation in brackish water, and may be hypo-osmotic in sea water, maintain the highest concentrations in fresh water. The lowest concentrations are found in those forms where the cells themselves come directly in contact with the environment as in the Protozoa, sponges, and coelenterates.

In the animals with extracellular fluids, sodium and chloride ions always account for a large part of the total osmotic pressure and as a result of the low concentrations of these ions in the water, large gradients are maintained. The potassium concentration of the blood is also higher than that in the water and in *Astacus* the same is true for all the other blood ions. In other freshwater animals accurate comparisons between the concentration of the divalent ions in blood and water have not been made and so it is not possible to generalize about these. However, it is clear that in all freshwater animals the processes of osmo-

TABLE II
THE COMPOSITION OF THE BODY FLUIDS OF SOME FRESHWATER ANIMALS[a]

Animal	Total concentration	Na	K	Ca	Mg	Cl	SO_4
Crustacea							
Telphusa fluviatilis (*12*)	695	—	—	—	—	301	—
Eriocheir sinensis (*35*)	645	—	5	10	3.5	282	—
Astacus fluviatilis (*35*)	480	—	5.2	10.4	2.7	194	—
(river water)	12	—	0.05	0.7	0.4	0.3	—
Teleost							
Coregonus clupeiodes (*26*)	315	141	3.8	5.3	1.7	117	2.3
Amphibian							
Rana esculenta (*36*)	261	104	2.5	2.0	1.2	74	1.9
Insect							
Aedes aegypti (*37, 38*)	287	110	3	—	—	43	—
Annelid							
Lumbricus terrestris (*39*) (in tapwater)	184	—	—	—	—	47	—
Mollusks							
Limnea peregra (*40*)	130	—	—	—	—	—	—
Anodonta cygnea (*40, 41*)	46	15.5	0.5	8.4	0.2	11.7	0.8
Coelenterates							
Hydra viridis (*42*)	40–50	—	—	—	—	—	—
Pelmatohydra oligactis (*42*)	—	2.3	14	—	—	0.15	—
Porifera							
Spongilla sp. (*43*)	50–60	—	—	—	—	—	—
Protozoa							
Spirostomum ambiguum (*44*)	50	1	7	—	—	0.6	—

[a] Concentrations of ions in millimoles per liter. Total concentrations in milliosmoles per liter equivalent sodium chloride solution.

regulation involve the uptake of sodium, chloride, and potassium against a concentration gradient and the removal of the water taken up by osmosis.

We may now turn our attention to the mechanisms by which anisosmotic states are maintained and the processes of osmoregulation carried out. We have established that some, at least, of these mechanisms have developed from those present in isosmotic forms. Of these, perhaps the most important are the mechanisms associated with the elaboration of the excretory fluid. This fluid, which may be produced by both hypo- and hyperosmotic animals serves for the removal of water and inorganic ions. Other mechanisms involve the uptake of ions, the secretion of ions (other than via the excretory fluid), and the absorption of water against an osmotic gradient.

III. The Mechanisms of Osmoregulation

A. The Function of the Excretory Organs in Osmoregulation

1. *The Composition of the Urine in Marine Animals*

(*a*) *Water.* All marine animals with excretory organs produce a liquid urine, whether the body fluid is isosmotic with sea water or not. In the hypo-osmotic forms this is a source of water loss, additional to that due to exosmosis and will, hence, tend to *oppose* the maintenance of an osmotic pressure difference.

(*b*) *Inorganic Ions.* In the marine invertebrates the excretory fluid is almost always isosmotic to the blood—the only known exception to this is the saline mosquito larva, *Aedes detritus* (Table III). The fluid contains all the major inorganic ions and the ratio of the concentration of an ion in the urine to that in the blood (the *U/P* ratio) generally approaches unity in the case of sodium, potassium, calcium, and chloride. Two examples of significant departures from this are the *U/P* ratios of 0.64 for calcium in *Homarus* and 0.78 for potassium in *Carcinus* and these low ratios are reflected in the higher concentrations of these ions in their blood (relative to sea water). In the same way, animals with low magnesium and sulfate concentrations in the blood, like *Carcinus* and *Palaemon*, have high *U/P* ratios for these ions. In *Carcinus*, it is likely that the whole of the magnesium and sulfate entering the animal by diffusion is removed in the excretory fluid (*26*). In the hypo-osmotic crustacea, like *Palaemon*, the elimination of divalent ions in the urine makes a significant contribution to the osmoregulatory processes, since these ions account for over 10% of the total osmotic pressure of sea water. We can not yet be certain of what percentage of the total monovalent ion secretion in these animals occurs in the urine. From analyses of the urine of *Palaemon* (32) it would appear that the proportion of sodium and chloride to magnesium and sulfate in the urine is approximately the same as the proportion in which these ions might be expected to enter the animal by diffusion (calculated from their respective concentration gradients and mobilities). It is, therefore, possible that in this species the removal of ions, necessary to maintain the low osmotic pressure, is effected solely through the urine and no extrarenal secretion takes place.

TABLE III

THE COMPOSITION OF THE URINE OF SOME MARINE ANIMALS[a]

Animal	Freezing-point depression	Na	K	Ca	Mg	Cl	SO_4
Mollusks							
Eledone cirrosa (*23*)	1.00	1.02	0.90	0.87	0.89	0.97	1.36
Crustacea							
Homarus vulgaris (*23*)	1.00	0.99	0.91	0.64	1.80	1.01	1.59
Carcinus maenas (*26*)	1.00	0.95	0.78	0.94	3.90	0.98	2.24
Eriocheir sinensis (*35*)	1.02	—	1.14	0.93	2.21	1.14	—
Palaemon serratus (*28, 31*)	1.00	0.82	0.86	0.95	6.70	1.06	3.80
Insect							
Aedes detritus (*45*)	3.30	—	—	—	—	—	—
Elasmobranchs							
Raia stabuliforis (*32, 46*)	0.93	—	9.8	13.8	70	1.1	422
Squalus acanthias (*46, 47*)	0.95	—	3.9	3.5	9.6	1.0	—
Teleosts							
Myoxocephalus octodecimspinosus (*48*)	0.95	—	—	—	18–77	0–0.8	~20
Lophius piscatorius (*49, 50*)	0.80–0.93	0.15	0.62	3.2	7.5–105	0.03–1.1	32

[a] The figures represent the ratio of the concentration in the urine to that in the body fluid (*U/P* ratio).

Among the vertebrates, the urine of both the marine teleosts and elasmobranchs is either isosmotic or slightly hypo-osmotic to the blood. The *U/P* ratios for magnesium and sulfate are especially high and, as in the invertebrates, the urine is the only means for their secretion (*51*). Calcium and potassium are also often more concentrated in the urine than in the blood but in the marine teleosts the sodium concentration is low and chloride is almost, if not entirely, absent (*48, 52*). In these animals sodium, chloride, and potassium must be excreted extrarenally (*51*).

2. *Composition of the Urine of Freshwater Animals*

(*a*) *Water.* Sources of water loss in freshwater animals, other than through the urine must be very small and it is generally assumed that the rate of urine production is equal to the rate of osmotic uptake of water. The removal of water, as urine, thus serves an important osmoregulatory function.

TABLE IV
THE COMPOSITION OF THE URINE OF SOME FRESHWATER ANIMALS (*U/P* RATIOS)

Animal	Freezing-point depression	Na	K	Ca	Mg	Cl	SO_4
Crustacea							
Telphusa fluviatilis (*53*)	1.00	—	—	—	—	—	—
Astacus fluviatilis (*35*)	0.11	—	0.12	0.26	0.46	0.05	—
Eriocheir sinensis (*35*) (in fresh water)	1.02	—	1.37	0.47	0.21	0.95	—
Annelid							
Lumbricus terrestris (*39*) (in tapwater)	0.19	—	—	—	—	0.07	—
Insect							
Aedes aegypti (*37*)	0.11	0.05	8.0	—	—	—	—
Mollusks							
Anodonta cygnea (*40, 54*)	0.60	—	—	0.75	—	0.55	—
Limnea peregra (*40*)	0.70	—	—	—	—	—	—
Teleosts							
Ameirus nebulosus (*55*)	0.05	—	—	—	—	—	—
Anguilla rostrata (*51*)	0.14	—	—	—	—	—	—
Elasmobranch							
Pristis microdon (*55a*)	0.10	—	—	—	—	0.03	—
Amphibia							
Rana esculenta (*56*)	0.08–0.13	0.02	0.38	0.39	0.33	0.02	0.26

(*b*) *Inorganic Ions.* The freshwater vertebrates produce a very dilute urine (Table IV.). In these animals, the older view stating that water is taken up osmotically through the semipermeable body surface and secreted again in the urine (*57*), is approximately correct. This simple idea cannot, however, be applied to the invertebrates. Among the crustacea, for example, three species from fresh water [*Telphusa fluviatilis* (53); *Eriocheir sinensis* (53); and *Palaemonetes antennarius* (58)] have been found with a urine isosmotic to the blood, whereas it is only in the crayfishes that a dilute urine has been found. In the mollusks, too, the urine concentrations are as high as 60 or 70% of the blood although in some other invertebrate groups, species may be found where the urine concentrations are almost as low as the vertebrates [for example, in *Aedes aegypti* (37); and in *Lumbricus terrestris* (39)]. In all the forms producing a dilute urine, a considerable amount of osmotic work must be done during the formation of this fluid.

The ionic composition of the urine varies with its osmotic pressure. Sodium and chloride are the most important ions osmotically, and in forms with an isosmotic urine, like *Eriocheir* (35), the *U/P* ratios for these ions are not far from unity, whereas in the frog, where the urine is very dilute, these ratios may be as low as 0.02. The *U/P* ratios for the other ions is usually between 0.1 and 1.0 whatever the total concentration, so that a small loss of these ions always occurs.

Despite the diversity in the composition of the urine of animals from both marine and freshwater environments, it is possible to come to some conclusions of general validity with regard to the function of the excretory fluid in osmoregulation.

In marine organisms showing hypo-osmotic regulation, the production of urine *assists* the processes of osmoregulation by removing magnesium and sulfate and, to a varying degree, the other inorganic ions which enter by diffusion. The water removed at the same time *impedes* regulation and adds to the water lost by osmosis.

Among the animals of fresh and brackish water which show hyperosmotic regulation, the formation of the excretory fluid *assists* by removing water which enters by osmosis, but *impedes* regulation, to a varying extent, by removing inorganic ions and thus increasing the total salt loss.

3. *The Formation of Urine in the Vertebrates*

The urine, as voided by the animal, is generally a final product resulting from a series of operations which determine its composition. It is important to establish the site of these operations within the vertebrate kidney and to localize the centers of active transport and osmotic work. Much of our knowledge of the functioning of the kidney stems from investigations on the mammals (59), but we shall consider this only inasmuch as it is relevant to an understanding of renal function in the aquatic vertebrates and with respect to the osmoregulatory processes. The work of H. W. Smith, E. K. Marshall, and their associates

(*60, 61*) has been largely responsible for our understanding of the comparative physiology and evolution of the vertebrate kidney from the standpoint of water and salt balance.

The basic structural unit of the excretory organ is the nephron. This consists essentially of a tube which is composed of a single layer of cells and which terminates at one end in the renal corpuscle and at the other end joins a collecting duct into which many other tubules enter. Many collecting ducts unite to form the ureter, so that the whole kidney contains many thousands of nephrons. The renal corpuscle is a capsule formed by a dilation of the proximal end of the nephric tubule and into this projects a tuft of capillaries derived from the arterioles of the renal artery (the glomerulus). The tubule itself is differentiated along its length into a number of segments which may vary both in number and structure in different vertebrate groups. In the amphibia, for example, the renal corpuscle is followed by a short neck segment, a proximal convoluted tubule, characterized by cells with a pronounced brush border (this segment is present in all vertebrates), a short intermediate segment, and finally a distal convoluted segment.

In the last century the theory had been advanced that the glomerulus was a filtering device by which blood was filtered under pressure through the glomerular epithelium into the lumen of the capsule, and hence down the tubule to the collecting duct. The experimental proof of this theory, mainly due to the classical studies of A. N. Richards and his associates, rested on the demonstration that: (*a*) an increase in blood pressure in the renal vessels of the rabbit resulted in an increase in urine flow (*62*); (*b*) the pressure in the glomerular capillaries, in the frog, was greater than the colloid osmotic pressure of the blood, which would oppose the water movement (*63*); and (*c*) fluid, extracted from the capsule of the frog and *Necturus,* contained the smaller solute molecules of the blood, such as chloride, glucose, urea, and potassium, in approximately the same concentrations as in the blood but no protein was present (*64, 65*). The filter will allow the passage of solutes of 4 mμ but not of 6–7 mμ in diameter.

This method of filtration for the separation of the excretory fluid is an economical one, since the only work done in its formation is by the heart in maintaining the necessary pressure in the capillaries.

In the amphibia, the urine is very dilute and often almost chloride-free and so the glomerular filtrate is considerably modified during its passage down the nephric tubule. This could take place either by the reabsorption of the ions of the filtrate or by the addition of water, either process requiring the presence of active transport systems in some part of the tubule. The examination of fluid samples withdrawn from different regions of the tubule (*66*), has revealed the location and nature of these processes. Measurements of the vapor pressure and the chloride concentration in the tubule fluid of the frog and *Necturus* (*67*) (Fig. 1) show that the filtered fluid remains isosmotic throughout the length of the proximal tubule and the intermediate segment. In the distal tubule the chloride concentration is greatly reduced and the total concentration also drops markedly. The distal tubule is therefore the site of the urine dilution. These measurements do not enable us to distinguish between dilution by the addition of water or by the removal of chloride. A study of the fate of filtered glucose (*68*) allows this to be done. This substance is normally strongly reabsorbed in the proximal tubule, but when the active transport of glucose is abolished by the action of phlorizin, the glucose concentration at the end of the proximal tubule is some 20–30% greater than in the plasma and by the end of the distal tubule the concentration has reached 2–3 times the plasma value (*69*). These results suggest that instead of water being added to the tubule fluid during its passage down the nephron, water is, in fact, reabsorbed in both the proximal and the distal tubules. Confirmation of this conclusion comes from studies on the normal, unpoisoned kidney by the clearance technique—a method which is extensively used to measure glomerular filtration rate and to demonstrate the occurrence of tubular secretion or reabsorption of any filtered substance. The ratio of the concentration of any filtrable substance in the urine to its con-

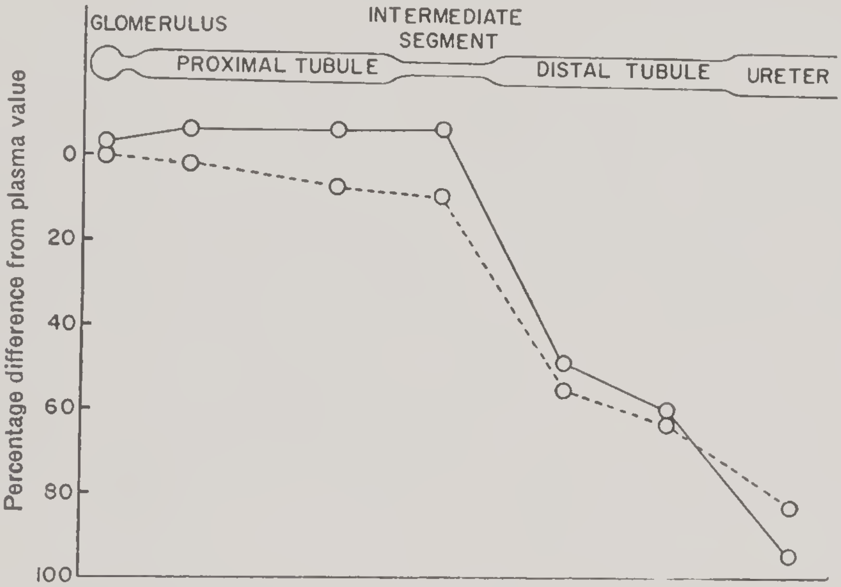

FIG. 1. The concentration of the tubule fluid in different regions of the nephron of *Necturus*. KEY: ○———○, chloride; ○– – – –○, total osmotic pressure. The points represent the mean values of a number of measurements and are shown as a percentage difference from the mean plasma values. [Based on data from Walker *et al.* (*67*).]

centration in the plasma (U/P ratio) multiplied by the rate of urine production (V) measures the clearance of the substance (UV/P) and represents the volume of plasma cleared of this substance in unit time. The clearance of a substance which is neither secreted nor reabsorbed by the tubules is a measure of the glomerular filtration rate. The polysaccharide, inulin, is widely used for this purpose (*68, 70*).

The application of the inulin clearance method to the frog (*71, 72, 73*) (Table V) shows on the average some 45% of the filtered water is reabsorbed and this is in good agreement with that calculated from the glucose measurements and, further, direct measurements of the inulin concentration at the end of the proximal tubule (*74*) confirm the removal of 20–30% of the water in this region. Since water is reabsorbed in the proximal tubule and yet the fluid remains isosmotic and the chloride concentration is unchanged, it follows that chloride (and sodium, as this is equally important osmotically) must also be reabsorbed in this tubule as well.

TABLE V

THE RATE OF URINE FLOW AND XYLOSE OR INULIN CLEARANCE IN SOME VERTEBRATES

Animal	Urine flow mean and range (ml./kg./day)	U/P ratio mean	Mean clearance rate (ml./kg./day)	Substance cleared
Elasmobranch				
Squalus acanthias	12.6	3.9	49	xylose (*92*)
		+25%	—	inulin (*93*)
Marine teleost				
Myoxocephalus octodecimspinosus	3.3 2.4–4.2	4.2	13.6	xylose (*82*)
Freshwater teleost				
Ameiurus nebulosus	153–326	1.25–1.90	240–427	xylose (*60*)
	41–82	—	—	—(*55*)
Amphibia				
Rana catesbiana	422 192–670	1.8	751	inulin (*71, 72*)
Mammal				
Man	normal 21 10–300	120	2550	inulin (*100*)

The combined results of these experiments gives us a clear picture of the role of the tubular segments in osmoregulation. The proximal tubule reabsorbs water, sodium, and chloride but the process is an isosmotic one and requires little or no work for its accomplishment. This process has no direct osmoregulatory function but may form an important variable in the finer adjustment of the salt content of the blood.

The distal tubule, on the other hand, is also the seat of water and sodium chloride reabsorption, but the transport of both sodium and chloride is against a large concentration gradient and results in the formation of a strongly hypo-osmotic fluid. It is here that the bulk of the osmotic work is done in producing the final urine.

The utilization of the most distal part of the nephric tubule for the diluting operation is not without significance. After the reabsorption of sodium chloride has taken place, the fluid must either pass into a duct which is relatively impermeable to water or else continuous osmotic work must be done on the fluid to maintain the low osmotic pressure.

The structure of the nephron is basically the same in all vertebrates and we can interpret the function of its parts in the kidney of the other aquatic vertebrates in the light of our knowledge of their role in the Amphibia. In the freshwater teleosts the basic design is much the same. Glomeruli are numerous and the tubule is divided into both proximal and distal convoluted regions (*61, 75*). The rate of urine formation in the catfish, *Ameirus* (Table V) and in many other species (*76*), is almost as high as in the frog, and the fluid is strongly hypo-osmotic to the blood. Xylose clearance in the catfish (Table V) shows also that the water reabsorption by the tubules is of the same order as in the frog. At present we know little more of the renal physiology of these fish, but salts must be reabsorbed in the tubule and, by analogy with the amphibian, this probably takes place in the distal convolution.

In the marine teleosts the conditions are very different. All water which is excreted in the urine, must be made good by the absorption of water against the osmotic gradient and this fact is reflected in the very low rate of urine production—often only some 2% of the rate in freshwater fishes (Table V) (*76*). The structure of the nephron also shows some very significant differences. Generally the distal segment is absent (*75*). The total glomerular filtration surface is smaller than in the freshwater forms (*77*) and in some species the glomeruli may be greatly reduced or completely absent (*60, 78, 79*). The reduction in filtration capacity can undoubtedly be correlated with the low rate of urine flow (*80*) although it cannot necessarily be argued from this that the vertebrate glomerulus evolved in the first place as an adaptation to freshwater life (*61, 81*). In the completely glomerular marine fish, *Myoxocephalus octodecimspinosus,* clearance studies (*52, 60, 82*) show that some 75% of the filtered water is reabsorbed (Table V) and from the urine analysis (Table III) it is clear that, in addition, practically all the chloride is reabsorbed, whereas both magnesium and sulfate are secreted.

In the aglomerular teleosts, like *Lophius piscatorius,* we find a striking similarity between the composition of their urine and that of the glomerular forms (Table III). In function, the kidney differs only in that it is unable to excrete those substances which, in the glomerular kidney are excreted solely by filtration—this especially applies to carbohydrates, like xylose, glucose (*83*), and inulin (*70*). The urine resembles that of the glomerular marine teleosts in the low concentration of sodium and chloride and the high concentrations of magnesium and sulfate (*48, 50, 52*) and a total concentration slightly below that of the blood.

The water of the urine in the aglomerular forms is not made available by filtration and therefore it must come through the tubule wall. It may follow passively the secretion of magnesium and sulfate into the tubule but, since the urine is normally slightly hypo-osmotic to the blood, the possibility of some active water secretion must be considered. The process bears some resemblance to the production of a hypo-osmotic fluid within cysts formed from pieces of proximal tubule from chick mesonephros in tissue culture (*84, 85*). Treatment of the sculpin, *Myoxocephalus* with large doses of phlorizin abolishes glomerular function (*86*) but, nevertheless, urine production may proceed at about the normal level. This important observation shows that the production of a fluid urine by secretion is not a special property which is confined to the aglomerular kidney.

The importance of the role of the urine in marine teleosts for the removal of magnesium is further revealed by an analysis of the diuresis which occurs when these fish are disturbed by handling. Urine flows greatly in excess of normal occur, and there is little doubt that this results from increased ingestion of sea water, since it may be prevented by the obstruction of the gastrointestinal tract (*87*). The diuretic urine is characterized by a high chloride content (*88*) and there is a strong correlation between the magnesium and chloride concentrations (*50*). An increase in the magnesium or sulfate content of the blood exhibits a marked diuretic effect (*89*) and, in the eel (*51*) the injection of magnesium chloride into the gut leads to the excretion of these ions in the urine. There can be no doubt that the concentration of magnesium (and probably, sulfate as well) in the blood is carefully regulated by the activity of the kidney.

In the sculpin, the increase in water secretion at the start of diuresis is due to an increase in the glomerular filtration rate but as the rate of urine flow increases, water reabsorption in the tubules is also reduced (*82*)

Although the structure of the teleost nephron may be related in a broad sense to the type of habitat in which the animal lives (*80*) there is no correlation between the structure of the nephron and the capacity of the fish to adapt itself to marine or freshwater conditions (*90*). Thus *Fundulus* and *Anguilla* can live in both fresh and salt water but the nephron of the former consists solely of a glomerulus and a proximal tubule, whereas the eel has a nephron typical of the freshwater teleost. Of especial interest is the freshwater pipefish, *Microphis boaja,* which has a typical marine aglomerular kidney (*90*).

Characteristic of the kidney of the marine elasmobranch are the large glomeruli and a filtration surface as large as that of the freshwater teleosts. Despite this, the rate of urine production is low, though it is several times greater than in the marine teleosts (Table V) (*46, 51, 91*). The tubules are divided into proximal and distal regions. From xylose and inulin clearance studies (*92, 93*) (Table V) and analyses of inorganic ions in the urine (Table III) important similarities with the processes of urine formation in the glomerular teleosts are revealed. Some 75% of the filtered water is reabsorbed together with chloride but magnesium and sulfate are secreted. The chloride concentration in the urine is, however, never reduced to the low values found in the teleost and the urine serves as an important route for the removal of chloride (and presumably sodium as well) in these animals. Urea, on the other hand, is strongly reabsorbed—the U/P ratio may be as low as 0.07—and this plays a vital role in the retention of urea in the blood (*46*). It was previously thought that the reabsorption of urea, which occurs only in the elasmobranchs, takes place in a special segment but it now seems unlikely that such a segment does in fact exist (*94*). It is possible that the balance between salt and urea in the blood is maintained by the degree of the reabsorption of these substances in the tubules.

The freshwater elasmobranchs are characterized by large and numerous glomeruli and a urine output of the same order as that of the freshwater teleosts. The blood contains considerably less urea than the marine forms (*95*) but this is still reabsorbed by the kidney. Salts are also strongly reabsorbed and the total osmotic pressure of the urine is as low as in the freshwater teleosts. Nothing is known of the sites of reabsorption or secretion within the tubules of any elasmobranch kidney.

The predominantly terrestrial vertebrate groups do not strictly come within the scope of this article, since in these animals diffusion of salts and water between the body fluid and the environment does not take place and there is no question of the maintenance of osmotic pressure differences. Maintenance of the constancy of the composition of the body fluids, however, is of great importance, especially in the mammals and we find the kidney intimately concerned with the regulation of water and salt balance.

The most urgent problem for those animals out of direct contact with water is the economical use of this substance in excretion. In birds and in reptiles from dry environments very little urine is produced and is often in a semisolid form, due to the presence of precipitated uric acid. The low rate of urine production is achieved, as in the marine teleosts by a great reduction in glomerular activity compared with that of a typical amphibian (*61*), although no aglomerular forms have been found. The urine is isosmotic or, perhaps, slightly hyperosmotic in the birds, but it is only in the mammals (among the vertebrates) where the ability to produce a hyperosmotic urine is well developed.

As with the birds and reptiles, the mammals produce urine at a relatively low rate, yet the characteristic feature of these animals is the very large glomerular filtration rate (Table V). In an average man, where some two million nephrons are engaged in the process of urine formation, filtration proceeds at a rate of some 180 liters a day—a rate which, weight for weight, is far greater than that found in the freshwater fish or Amphibia. The enormous reduction in the amount of the filtrate as it flows through the nephrons is brought about largely by the reabsorption of water, isosmotically with sodium and chloride, which occurs in the proximal tubule (*96*). This process removes over 80% of the filtered water, compared with 20–30% in the frog. In man, the proximal reabsorption will reduce the 180 liters of fluid originally filtered to some 27 liters in a day and, in fact, a man suffering the extremes of diabetes insipidus may actually produce urine at this rate. It is likely that, in this condition, the only water reabsorption taking place during the formation of the final urine occurs in the proximal tubule. In a normal man the rate of urine flow is much less than this (Table V) and further reabsorption has taken place. In the current theory of water and salt relations of the mammalian kidney (*59, 97*), the filtered fluid, after proximal reabsorption, undergoes, first of all, a process of osmotic equilibration in the intermediate segment (loop of Henle). The fluid, on entering the distal tubule is then diluted by the reabsorption of sodium and chloride against a large concentration gradient. This is analogous to the process occurring in the distal tubule of amphibians and accounts for the production of the very dilute urine which is found during water diuresis. In the normal and in the dehydrated animal further water reabsorption takes place, probably by two separate processes. The first process, operating in the distal tubule, can restore the tubule fluid to the isosmotic condition by the uptake of water in the direction of the osmotic gradient. This requires no work and the uptake is controlled by the action of the antidiuretic hormone (see below)—its absence explains the high rate of urine flow in diabetes insipidus. The second process, which must be located further down the nephron [possibly in the collecting ducts (*59*)] brings about an additional water reabsorption and concentration of the urine, so that the osmotic pressure of the final fluid may be several times greater than that of the blood. This is an active process, since the water is reabsorbed against the osmotic gradient and the amount of water which can be removed is limited to a rather small maximum value for a given volume of glomerular filtrate (*98, 99, 100*).

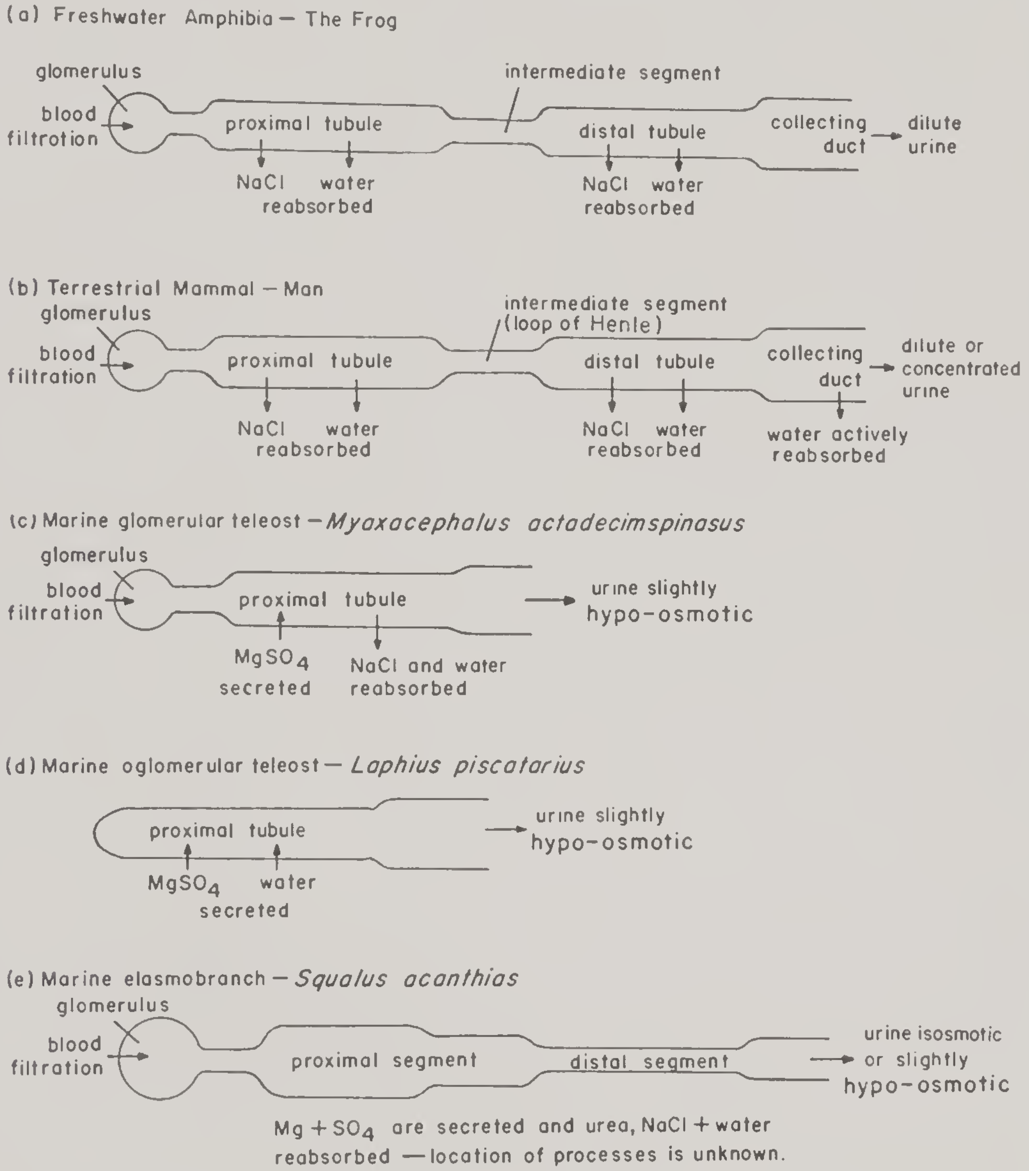
(a) Freshwater Amphibia — The Frog
glomerulus
blood filtrotion
proximal tubule
NaCl water reabsorbed
intermediate segment
distal tubule
NaCl water reabsorbed
collecting duct
dilute urine
(b) Terrestrial Mammal — Man
glomerulus
blood filtration
proximal tubule
NaCl water reabsorbed
intermediate segment (loop of Henle)
distal tubule
NaCl water reabsorbed
collecting duct
dilute or concentrated urine
water actively reabsorbed
(c) Marine glomerular teleost — *Myoxocephalus octodecimspinosus*
glomerulus
blood filtration
proximal tubule
$MgSO_4$ secreted
NaCl and water reabsorbed
urine slightly hypo-osmotic
(d) Marine aglomerular teleost — *Lophius piscatorius*
proximal tubule
$MgSO_4$ water secreted
urine slightly hypo-osmotic
(e) Marine elasmobranch — *Squalus acanthias*
glomerulus
blood filtration
proximal segment
distal segment
urine isosmotic or slightly hypo-osmotic
Mg + SO_4 are secreted and urea, NaCl + water reabsorbed — location of processes is unknown.

FIG. 2. A schematic representation of the structure of the nephron in some vertebrates. The location of the main processes associated with osmoregulation are indicated, where these are known. [Diagrams based on data from H. W. Smith (*59*); Marshall (*60*); and Kempton (*94*).]

If this theory proves to be substantially correct, we can see that the mammalian nephron differs in function from that of the amphibian nephron only in a quantitative manner, with the exception of the final concentrating process. This process is clearly an evolutionary development, correlated with the need for water conservation and the retention of urea as the major nitrogenous excretory product.

A schematic representation of the location within the vertebrate nephrons of those processes especially concerned with osmoregulation are shown in Fig. 2. In all but the aglomerular teleosts, the isosmotic urine is formed by a combination of filtration and proximal reabsorption. In freshwater forms osmotic work is done by the distal tubule cells by the active reabsorption of sodium and chloride. The marine forms are characterized by the proximal active transport of magnesium and sulfate. In the elasmobranch nephron a specialized process is present for the active reabsorption of urea.

4. *The Formation of Urine in the Invertebrates*

The excretory organs of the invertebrates are varied in character and structure. As a group, they are as heterogeneous as the vertebrate organs are homogeneous and there are no evolutionary trends to help us bridge the gap between the different phyla. In many cases, however, the organs are amenable to experimental study in the same way as the vertebrate nephron, although at present such studies are not extensive. Investigation has been confined to the annelids, mollusks, and arthropods.

In the majority of the annelids the excretory organs consist of segmentally arranged nephridia. These are tubules which open to the exterior at their distal end and, in the majority, terminate at the proximal end in an open ciliated funnel — i.e., the nephrostome. As the nephrostome opens into the coelomic cavity, coelomic fluid can pass directly to the exterior. Fluid expelled from the nephridia is presumably replaced by filtration of blood through the walls of the blood vessels into the coelom and, in this sense, the excretory fluid may be regarded as having originated as a blood filtrate. The coelomic fluid only differs slightly from the blood. In the earthworm, for example, the former is isosmotic or slightly hyperosmotic and the chloride concentration slightly greater than that of the blood (*39*). Nothing is known of the composition of the excretory fluid in the marine forms but the general similarity of the blood with sea water suggests a relatively unchanged coelomic fluid must be excreted.

The earthworm, however, whose osmotic behavior is typical of a freshwater animal, excretes a fluid considerably more dilute than the blood (*39*) (Table IV). Measurements of freezing point depression of the fluid from various parts of the nephridium (*101*) show that the fluid is isosmotic throughout the long narrow tube and becomes hypo-osmotic in the distal segment (the wide tube) and possibly to a slight extent in the preceding segment (the middle tube). The wide tube is clearly the site of the osmotic work and this brings to mind the distal reabsorption in the amphibian nephron. It is not possible yet to distinguish between salt reabsorption or water secretion as the responsible operation.

In the mollusks the excretory system resembles that of the annelids in that the coelomic fluid may pass directly to the exterior. The coelom, however, is very much reduced in extent by the expansion of the hemocoele which restricts it to the pericardium, surrounding the heart. The excretory organs are a pair of coelomoducts (a single one in the higher gastropods) which open from the pericardium to the exterior and their fluid probably passes into the pericardium by filtration through the thin walls of the auricles. Like the annelids, most marine species probably excrete coelomic fluid largely unchanged. The cephalopods, like *Eledone* (Table III), show marked powers of ionic regulation and this is reflected in ionic differences in the urine (*23*). Nothing is known of where such differences are generated.

In the freshwater lamellibranch, *Anodonta*, the possibility of filtration forming the initial step in urine formation has some experimental basis with the demonstration that the blood pressure exceeds the difference in colloid osmotic pressure between the blood and pericardial fluid (*40*). In this animal the pericardial fluid is isosmotic with the blood but its concentration falls to about 60% during its passage through the tubule—a fall which is paralleled by a drop in the chloride concentration (*40*). Inulin clearance (10 ml./kg./hour) and urine production rate (19 ± 11 ml./kg./hour) measurements (*102*) do not definitely exclude water secretion in the tubule but it is clear that little water movement is involved.

Restriction of the coelom to small dimensions is also characteristic of the crustacea. Here it is represented by a pair of small sacs, from each of which a tubular excretory gland arises and opens to the outside. In the higher crustacea, like the crabs and lobsters, the excretory tubule has a complicated glandular portion, known as the green gland or labyrinth, and the lumen of this part is much divided by septa lined with a epithelium of secretory cells. In the marine forms, fluid from the labyrinth is discharged into a large bladder which opens to the exterior through an excretory pore (*103*) (Figure 3). As in the annelids and mollusks, coelomic fluid can be passed to the exterior through the tubule, but it has long been a subject of speculation as to how fluid enters the coelom sac. Both the coelom sac and the labyrinth are well supplied with blood and the possibility of filtration through the wall of the coelom sac has always had its attractions. The composition of the excretory fluid of marine forms, like *Homarus* and *Carcinus*

(Table III), would lend support to this hypothesis since the fluid resembles the blood closely (except for its higher concentrations of magnesium and sulfate) and is practically protein-free. The fact that the blood pressure in *Carcinus* exceeds, in most cases, the difference between the colloid osmotic pressure of blood and urine (*30*) adds further evidence, as does the suggestive (but hardly conclusive) demonstration that an increase in hydrostatic pressure in *Carcinus* may lead to a greater rate of urine production (*104*). In the lobster, inulin and creatinine, introduced in the animal, appear in the urine in the same concentration as they are present in the blood (*105*) this, again, suggests a non-selective filtration process and, further, that no secretion or reabsorption of water takes place in the excretory organ. The magnesium and sulfate *U/P* ratios, in the lobster, are both greater than unity (Table III) so these ions must be secreted into the excretory fluid at some stage during its formation. The secretory nature of the labyrinth wall suggests that this may be responsible for the divalent ion transport but no direct evidence is available to substantiate this.

There is little difference between the structure and function of the excretory organs of the marine crustacea and those of their freshwater relatives which produce an isosmotic urine (*15, 53*). In the crayfishes, however, and certain other freshwater species (*27, 103*) the excretory tubule may be much longer than in the typically marine forms. The crayfish has, between the green gland and the bladder, an additional canal which for most of its length has a much-folded wall containing glandular epithelial cells. For a long time the presence of this extra structure has been correlated with the ability of the animal to produce a dilute urine. Analyses of fluid from different parts of the excretory tubule show that, whereas the chloride concentration in the coelom sac and the labyrinth is the same as in the blood, it is reduced to less than half in the fluid from the middle of this canal (*103*) and this strongly supports the location of the diluting mechanism here. Dilution may occur by the reabsorption of sodium chloride but the possibility of water secretion in the canal has not been ruled out (*106*)—indeed, the whole concept of the applicability of the filtration-reabsorption theory to the formation of urine in the crayfish has been called into question (*107*). No final answer can be given at the present. The occurrence of filtration is suggested by measurements of blood pressure (*30*), the fact that the chloride concentration of the coelom sac is the same as the blood, (*103*), and by the appearance of creatinine and inulin in the urine after injection into the blood (*107*); but a more detailed analysis of this problem is required.

Of special interest with regard to theories of urine formation are the excretory organs of the insects. These consist of a number of closed Malpighian tubules which open at their distal ends into the gut at the junction of the mid and hind guts. Fluid is produced in the tubules and this passes into the intestine and rectum, where it may be greatly modified in composition, and finally discharged through the anus (Fig. 3). The Malpighian tubules are certainly secretory glands, like the tubules of the aglomerular fish, and there is no question of a primary filtration process being involved in the formation of the fluid. The formation of the urine in the insects shows a number of unique features. In the first place, the tubular fluid which is practically isosmotic with the blood contains large amounts of potassium (*108*). In the stick insect, for example, the concentration of potassium in the fluid is some 13 times that of the blood whereas it has less than half of the blood sodium level (*109*). Chloride and phosphate are also present but the greater proportion of the anions are still unknown. Another striking feature is the fact that the ions secreted into the tubule are largely reabsorbed again in the rectum. In the stick insect some 95% of the sodium and 80% of the potassium are reabsorbed, together with a large amount of water, and the resultant fluid is hyperosmotic to the blood (*110, 111*).

The aquatic insects, which are all thought to be derived from terrestrial forms, secrete a urine formed in the same manner. In the freshwater mosquito larva, *Aedes aegypti*, the Malpighian tubules secrete a fluid containing 88 mM potassium and 24 mM sodium (compared with 3 mM potassium and 87 mM sodium in the blood) and isosmotic with the blood (*37*). Both these ions are strongly reabsorbed in the rectum and the final fluid is very dilute (*45*): the main difference from the terrestrial insect being in the reduction in the extent of the distal water reabsorption. There is a striking parallel between the processes in the distal tubule which distinguish the freshwater amphibian from the terrestrial mammal and those in the insect rectum which characterize the animals from those environments. In the formation of urine in the insects, work is done both in the secretion of the Malpighian tubule fluid [mainly in the active secretion of potassium (*108*)] and in the reabsorption which occurs in the rectum.

The saline water mosquito larva, *Aedes detritus*, resembles terrestrial forms in that the Malpighian tubule fluid becomes slightly hyperosmotic in the intestine and the concentration is further increased in the rectum until, in sea water, it is some 3 times that of the blood (*37*). Active water reabsorption probably accounts for this concentration, but the possibility of salt secretion in the rectum must not be overlooked.

A diagrammatic representation of the processes of urine formation, as far as they affect osmoregulation, is shown in Fig. 3. There is little point in attempting any extensive generalizations among so diverse a group of organs, although some features are worth comment. Firstly a combination of filtration, secretion, and reabsorption in the formation of urine, as we find in the vertebrates, makes an acceptable hypothesis for

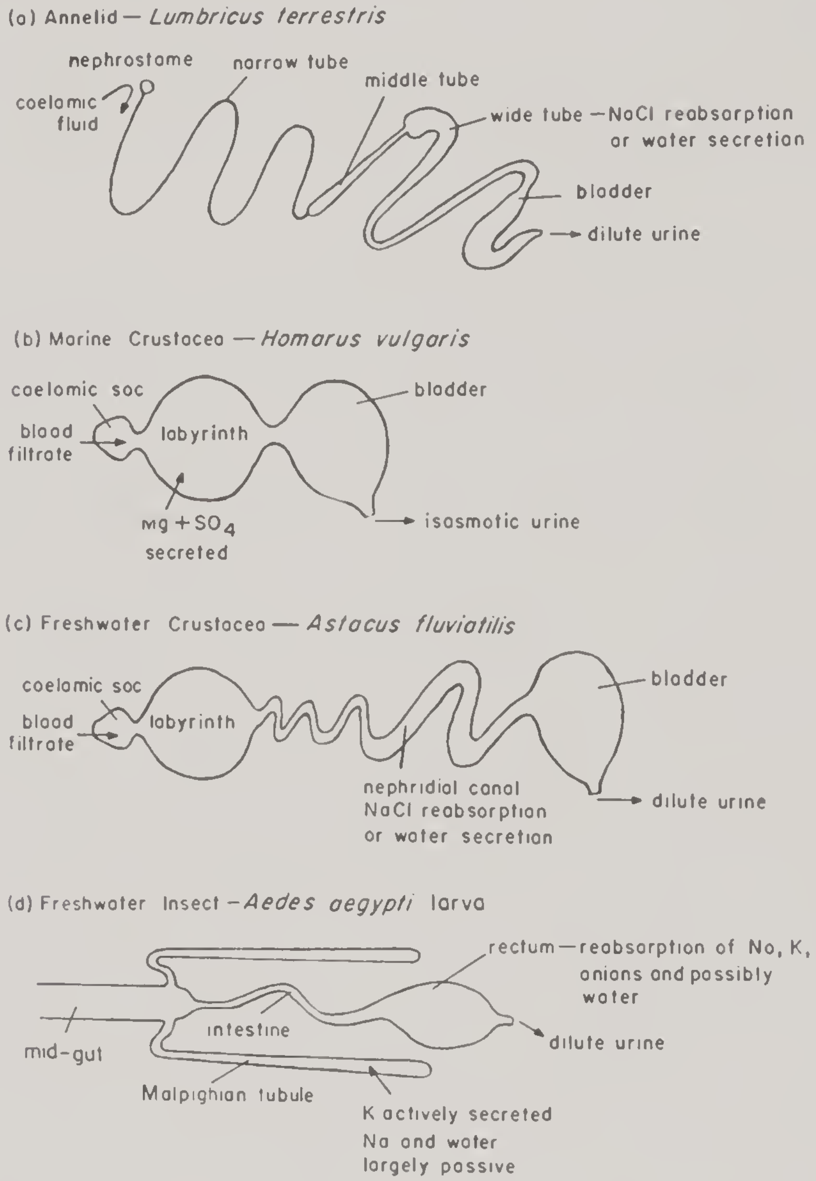
(a) Annelid — Lumbricus terrestris
nephrostome
narrow tube
middle tube
coelomic fluid
wide tube — NaCl reabsorption or water secretion
bladder
dilute urine
(b) Marine Crustacea — Homarus vulgaris
coelomic sac
bladder
blood filtrate
labyrinth
Mg + SO4 secreted
isosmotic urine
(c) Freshwater Crustacea — Astacus fluviatilis
coelomic sac
bladder
blood filtrate
labyrinth
nephridial canal NaCl reabsorption or water secretion
dilute urine
(d) Freshwater Insect — Aedes aegypti larva
rectum — reabsorption of Na, K, anions and possibly water
intestine
dilute urine
mid-gut
Malpighian tubule
K actively secreted Na and water largely passive

FIG. 3. A diagrammatic representation of the structure of the excretory organs of some invertebrates. The location of the processes associated with osmoregulation are shown, where these have been ascertained. [Based on data from Ramsay (*37, 45, 101*); Peters (*103*); Robertson (*22*).]

all the invertebrates which have been studied, with the exception of the insects. Secondly, in all the freshwater forms which produce a dilute urine, the excretory organ has some region near its distal end where the osmotic work of dilution is performed. The insects, which are predominantly a terrestrial group, parallel the mammals in the possession, by many forms, of a mechanism, located distally, for producing a urine more concentrated than the blood. Finally the Malpighian tubules of insects resemble, in certain respects, the aglomerular tubules of marine teleosts. The latter selectively secrete one cation, magnesium, whereas the former select potassium and in both, an increased concentration of the specific cation in the blood produces a great increase in urine flow.

Generally speaking, the invertebrate excretory organs have not received the thorough attention devoted to the vertebrate nephron and there remains a great deal of scope for future work in this field.

5. *Regulation of Salt and Water Content*

We cannot assume that the excretory fluid of any animal removes a constant amount of water or salts. The processes involved in urine formation are generally under physiological control and their variable activity is reflected in the composition and volume of the final urine. In this way the excretion of urine may assist not only in the primary processes of osmoregulation but also in buffering the osmotic pressure against abnormal changes in salt or water content.

Our knowledge of the regulation of water excretion is confined almost entirely to the vertebrates. Among the Amphibia, it has been known for a long time that frogs kept in a state of dehydration or in an isosmotic salt solution, produce only small amounts of urine (*112*). In fact, most amphibians in different degrees of hydration and dehydration show very large variations in the rate of urine production (*71, 73, 112, 113, 114*). These variations result from changes in the glomerular filtration rate (*71, 73*), and there is little doubt that in the amphibians this forms the major method for the regulation of water loss. Water reabsorption in the tubules is another variable factor (*14*) but there is no obvious relation between this and the glomerular filtration rate, so its role in water excretion is not yet clear. In the dehydrated condition, the urine volume may be further reduced by reabsorption through the wall of the bladder (*115, 116*).

The regulation of the urine volume by changes in the glomerular filtration rate is probably the primitive method of water control among the vertebrates (*117*). In the mammals it has only been recorded in the rabbit, and this is questionable (*59*). In the dog and rat, the state of hydration has no effect on the glomerular filtration rate except at extremes of dehydration and in adult man it is even more stable (*117*). Nevertheless large variations in the rate of urine production are common in man and other mammals and regulation of water excretion is achieved by changes in the degree of tubular water reabsorption. According to the modern theory of tubular function in the mammal, this takes place in the distal tubule and may well result from changes in the permeability of the tubule wall to water.

In the mammals the volume of water excreted is controlled by the antidiuretic hormone secreted by the neurohypophysis. Administration of this hormone during water diuresis results in an immediate reduction in urine flow and its effect is mainly, if not entirely, confined to the distal tubule reabsorption. The significance of this hormonal control, as far as water balance is concerned, is shown by the conclusive demonstration that one of the factors governing its release is the increase in the osmotic pressure of the blood plasma (*118*). Changes of the osmotic pressure in the order of 2% are effective and certain solutes, like sucrose, may evoke the response, as well as sodium chloride. The stimulus for the release comes from sensory receptors located in the hypothalamus.

Many other factors, including many emotional stimuli, may be responsible for the release of the antidiuretic hormone, but of particular interest is the possibility that the extracellular volume may exert a controlling action. Nothing is known of the receptors by which this action could be effected.

Among the other vertebrates, the role of the neurohypophysis in controlling water excretion is not so well established. Although in frogs and toads, increasing the blood osmotic pressure with sodium chloride or glucose leads to a reduction in urine production (*119*), the removal of the neurohypophysis in the frog has little effect (*120*), and hypophysectomized frogs and toads are still capable of reducing urine flow in response to dehydration (*121*). The injection of extracts of both mammalian and amphibian hypophyses into amphibians often leads to a marked antidiuretic response especially in the semiterrestrial forms, like the toad (*122, 123*). This effect is mainly due to reduction in the glomerular filtration rate (*124*) but it also has some effect on tubular reabsorption (*123*) and uptake from the bladder (*115, 116*). Whether these effects manifest themselves in the normal animal or not still requires investigation.

Practically nothing is known of water balance in the fishes. The fact that the glomerular filtration rate in the freshwater catfish (Table V) is much greater than that of the marine sculpin, together with the fact that the glomeruli of the marine forms may be greatly reduced or absent (*60*), suggest that a reduction in filtration rate is the most important renal response in fish, under conditions of dehydration, as it is in the Amphibia. Such a response would be of especial importance to the migratory fish, like the eel, which pass from fresh water to sea water and vice versa and change their rate of urine flow some 50-fold during the process.

The dogfish, in dilute sea water increases its rate of urine flow. Again this is effected by an increase in filtration rate (*125*) and is further evidence of the applicability of this primitive method of water regulation to the aquatic vertebrates.

Among the aquatic invertebrates, beyond the fact that certain freshwater animals, like the crayfish (*126*), reduce urine flow in isosmotic salt solutions and marine animals, like *Carcinus*, may increase urine production in dilute sea water (*8*), little quantitative data is available on the regulation of water excretion.

Our knowledge of the regulation of salt output in the excretory fluid of aquatic animals is surprisingly limited. Measurements of the salt concentration of the urine tell us little about the total salt excretion unless simultaneous determinations of the urine production rate are made. In the Amphibia, for example, the urine salt concentration of the frog may vary from 0.05 mM in the salt-depleted animal to a concentration isosmotic with the blood in the salt-loaded forms. However, in the latter case the rate of urine production is much lower than normal and the total salt secretion is not as great as appears at first (*126a*). The excretion of sodium varies with the glomerular filtration rate and also with the degree of reabsorption by the tubules. In the bullfrog, tubular reabsorption may vary between 94 and 99.5% and the extent of the reabsorption is not related to the filtration rate (*114*). The total sodium excretion results from a balance between filtration and tubular reabsorption, just as water excretion does, but little is known of the way the regulation of these processes is linked to the specific sodium requirements of the animal.

In aquatic insect larvae, *Aedes aegypti* and *Sialis lutaria,* the sodium concentration of the excretory fluid is greatly increased in animals kept in isosmotic salt solutions (*108*, *127*). In the mosquito larva, salt loading leads to an increase in the sodium concentration of the Malpighian tubule fluid and this is further increased as it passes through the rectum. Similarly the chloride concentration in the urine of the earthworm is increased, if the outside salt concentration is raised (*39*) and the same is true of the osmotic pressure of the urine of crayfish (*128*). This response of freshwater animals which normally produce a dilute urine, i.e., an increase in the salt concentration of the urine resulting from an increase in the salt concentration of the environment, may well be a general phenomenon, but to what extent this is concerned with regulating the salt content of the animal remains to be determined.

B. Mechanisms for Ion Absorption

In animals maintaining a hyperosmotic state, the total loss of ions due to diffusion through the body surface and removal in the urine is made good by absorption. In some animals this may take place in the gut, from salts present in the food, but in many animals special mechanisms, located in the more permeable regions of the body surface, have been developed. These mechanisms are especially well developed in freshwater animals (*16, 128, 129*) where ion absorption takes place against very large concentration differences; but they are also found in brackish water animals, like *Carcinus* (*8*) and even in some isosmotic marine animals where they assist in maintaining ionic differences in the blood (*26*). There are a few cases among the freshwater animals, like the eel in fresh water (*16*) and the alder fly larva (*130*) where mechanisms for absorbing ions from dilute solutions appear to be completely absent. Most uptake mechanisms are capable of absorbing sodium and chloride, and in some arthropods, like *Eriocheir sinensis* and *Aedes aegypti*, potassium can also be transported. As yet, there has been no convincing demonstration of the uptake of calcium, magnesium, or sulfate.

Despite the widespread occurrence of ion-absorbing mechanisms, there are a surprising small number of animals in which they have been localized with certainty and analyzed in detail. The processes of active transport are dealt with in an earlier chapter of this volume; we are concerned, here, with the location of these processes and the way in which they are regulated to meet the needs of the animal as a whole.

Amphibians can keep themselves in salt balance in very dilute salt solutions, even during starvation (*16, 131*). A frog, washed in distilled water loses chloride through the urine and the skin, but if it is then transferred to a dilute sodium chloride solution (about 1 m*M*) it takes up chloride and reduces the concentration to 0.01 m*M* or less (*16*) (Table VI). The uptake mechanism is located in the skin and, in fact, the isolated skin bathed in Ringer solution will continue to transport ions for many hours.

From sodium chloride solutions, the frog absorbs sodium and chloride together but the existence of separate mechanisms for each of these ions is demonstrated by the fact that chloride can be absorbed from calcium or potassium chlorides, without the cation, and that similarly sodium can be taken up from bicarbonate and sulfate solutions without the anion (*128*).

Use of the radioactive tracer technique reveals that ion absorption is occurring continuously in the normal, as well as in the salt-depleted animal (*131, 135*). A detailed analysis of ion transport in the isolated frog skin (*136*) shows that sodium ions are actively transported across the skin from the outside—a process which generates the observed potential difference across the skin—whereas chloride ions are transferred passively. In the intact frog, however, active transport of chloride will take place from dilute potassium chloride solutions (*137*) and, in some cases, chloride uptake is even found in dilute sodium chloride solutions, in addition to the more energy-consuming sodium transport (*137*). This again emphasizes the discrete nature of the two mechanisms, one of which (the chloride transport) is, apparently, inactive in the isolated skin.

TABLE VI

ION ABSORPTION BY SOME FRESHWATER ANIMALS

Animal	Maximum net uptake of chloride by salt-depleted animals from 1–3 m*M* NaCl (microequivalents/100 gm./hour)	Lowest outside concentration for balance in salt-depleted animals (millimoles/liter)	Total sodium influx in normal animals from 1–3 m*M* NaCl (microequivalents/100 gm./hour)	Ions absorbed
Crustacea				
Eriocheir sinensis (*9, 138*)	500	0.3	200–300	Na, K, Cl
Astacus fluviatilis (*16, 133*)	15	<0.005	10–20	Na, Cl
Teleost				
Goldfish (*9, 134*)	—	0.11	40	Na, Cl
Amphibia				
Rana esculenta (*128, 135*)	20	<0.005	22	Na, Cl

The rate of ion absorption is an important variable in the amphibians and is under physiological control. For example, the influx of sodium in the frog and toad from 3 m*M* sodium chloride solutions is about twice as great in animals previously salt-depleted by washing in distilled water (*135*). Furthermore, the sodium and chloride absorbing mechanisms may be activated independently. Thus in frogs depleted in chloride but not in sodium, the chloride uptake exceeds that of sodium, and conversely, if the animal is deficient in sodium but not in chloride, then the sodium uptake is the greater (*137*). This clearly demonstrates the presence of some form of internal regulation over the rate of ion absorption but the way in which this operates is not understood. Mammalian and amphibian neurohypophysis extract injected into amphibians increase the rate of sodium uptake (*121, 131, 138*) but whether the hypophysis plays any role in regulation in the normal animal cannot yet be decided.

The rate of ion absorption may also be influenced by the concentration of the outside solution. In the salt-depleted frog, the net uptake of chloride is about 10 times greater from a 10 m*M* sodium chloride solution than from one of 1 m*M* (*128*). In the isolated skin, the sodium transport increases, with increasing outside concentration up to about 40 m*M*, whereafter it is fairly constant (*139*).

Among the freshwater fishes, ion-absorbing mechanisms are generally present although their activity varies greatly in different species (*129*). They are well developed, for example, in the goldfish but relatively inactive in the ruff and perch. In the goldfish, the net uptake of chloride after salt depletion is of the same order as in the frog (Table VI) but here, the mechanism is not generally distributed over the skin but located in the anterior part of the animal, presumably in the gills (*129*). As with the Amphibia, absorption is confined to sodium and chloride ions and these are taken up by separate mechanisms. The uptake of sodium proceeds continuously in the normal fish and this is balanced by the normal sodium loss (*134*).

Among the invertebrates, the crab *Eriocheir sinensis*, in fresh water is known to possess great powers of salt uptake (*9*). The net absorption in salt-depleted animals (*9*) and the sodium uptake in normal crabs (*132*) is some 10 times that found in the frog and goldfish (Table VI). This high rate of absorption is correlated with the loss of salts by way of the isosmotic urine and the even greater loss by diffusion through the relatively-permeable gill surface—the latter accounting for about 86% of the total loss (*9*). The uptake mechanisms are located in the gills and these will continue to absorb ions for some hours after isolation (*140*). Potassium may be absorbed simultaneously with sodium but the potassium uptake does not affect the rate of sodium transport—this also points to the existence of separate mechanisms responsible for the transport of these ions.

In the freshwater crayfish, the production of a dilute urine and the relatively low permeability of the body surface, leads to a much smaller salt loss than in *Eriocheir* (*16*). Associated with this we find that the sodium influx and the net chloride uptake after salt depletion (Table VI) are much less than in the crab and more comparable with the uptake of these ions in the aquatic vertebrates. Undoubtedly, the reduction in the permeability of the body surface in freshwater animals is one of the most important factors in reducing the work which must be done in absorbing ions from their environment.

Certain aquatic larvae of insects, like those of the mosquito *Aedes aegypti*, and of *Chironomus* and *Culex*, possess anal gills, which are special structures concerned with ion absorption (*38*, *141*). The mosquito larva can absorb sodium, chloride, and potassium from dilute solutions (*37*) and the sodium uptake proceeds continuously in the normal larva, its rate being unaffected by the presence of potassium (*142*).

C. Mechanisms for Water Absorption

Animals maintaining a hypo-osmotic state must absorb water to balance the loss by osmosis and in the urine. Such a situation is found in all the marine teleosts, a few marine crustacea, like *Palaemon serratus*, and some marine insect larvae, like *Aedes detritus*.

The route by which the absorption of water takes place is only known with certainty in the marine teleosts. Here water is taken up from the intestine as a result of the ingestion of large quantities of sea water (*51*). The sculpin and the eel (in sea water) ingest some 50–200 ml. of sea water a day per kilogram body weight and from this, some 60–80% of the contained water is absorbed. The osmotic pressure of the ingested sea water is reduced in the stomach (probably by the addition of gastric juices) and then reduced still further in the intestine, where the absorption occurs, until it becomes approximately isosmotic with the blood. During the absorption, some solutes must also be removed, although magnesium and sulfate become several times more concentrated than in the original sea water. The degree of concentration of these ions roughly corresponds with the amount of water absorbed. Sodium, chloride, and potassium are not concentrated and are clearly, themselves, also absorbed (*51*). In the case of sodium and chloride, the concentrations may fall, not only below that of the ingested sea water, but also below that of the blood, so that active absorption of, at least, one of these ions must take place. The absorption of the water probably follows the rapid uptake of the univalent ions and there is no evidence of active water absorption. In any case, little osmotic work is done as the complete absorption is an isosmotic process. The mechanism resembles, in many respects, the absorption of sodium chloride and water that occurs in the proximal tubule of the amphibian and mammalian nephron.

There is no reason to suppose that the absorptive mechanism in the intestine of the marine teleost represents a special adaptation of the vertebrate intestine for the purpose of water absorption from sea water. In the mammalian intestine, tracer studies show that the intestine wall is freely permeable to sodium, chloride, and water in both directions (*143, 144*). The introduction of a hyperosmotic solution, such as sea water, leads to a movement of water from the blood to the gut until the solution becomes isosmotic (*145*). The fate of isosmotic mixtures of sodium chloride with magnesium chloride, sodium sulfate or magnesium sulfate, introduced in the dog intestine (*146, 147*), demonstrates the absorption of sodium and chloride to the exclusion of magnesium and sulfate. Water uptake occurs simultaneously and the solutions remain isosmotic. The concentrations of sodium and chloride may fall well below that of the blood and this may be prevented by metabolic inhibitors, like arsenite or mercuric chloride. The behavior of these salt mixtures in the mammal accords with the fate of ingested sea water in the marine teleost intestine and the simplest interpretation is that: (*a*) the intestine wall is relatively impermeable to magnesium and sulfate; (*b*) sodium and chloride are actively and rapidly absorbed; and (*c*) water is absorbed isosmotically, following the absorption of the univalent ions.

In the mammal, the question of active water absorption has always been in debate. Evidence for the passive absorption comes from a study of the behavior of autogenous serum introduced in the dog intestine (*148*). The absorption of salts always produces a slight osmotic gradient along which water movement can take place. Further, a detailed analysis of the absorption of salt solutions from the perfused intestine of the rat using tracer methods, shows that both sodium and chloride are actively absorbed and that the rate of water uptake can be interpreted in terms of the osmotic gradient and the rate of absorption of the solutes (*149*).

There seems no essential difference between absorption of salt solutions in the mammalian intestine and in that of the marine teleosts and, in fact, this behavior may be characteristic of the vertebrate intestine in general. Water absorption can thus proceed rapidly and with little osmotic work being done; but in the marine teleost the simultaneous salt uptake must be balanced, in the long run, by an equal salt secretion. It is in this process, where the energy required for the transport of water against the osmotic gradient, is ultimately used up.

Among the invertebrates which maintain a hypo-osmotic state, the site of water absorption is not clearly established. In the marine prawns, drinking has been observed but it is neither continuous nor extensive (*28, 150*). In *Carcinus*, where water absorption occurs to balance the loss of water in the urine, it takes place even when the mouth is blocked (*8*). In the normal animal, drinking is very limited, except during the molt (*20*) and it would appear that water uptake is an active process probably located in the gills (*26*). It is possible that a mechanism of this kind is of general occurrence in the marine crustacea and could account for water uptake in hypo-osmotic species, but proof of the active nature of the mechanism is still required.

The larva of the mosquito, *Aedes detritus*, in sea water, is markedly hypo-osmotic to the environment (*34*). The permeability of the integument is low and little water is lost by osmosis, but some water absorption must take place to replace fluid lost in the urine. Exchange of water and salts takes place mainly in the gut (*34*) and there is little doubt that, as in the marine teleost, water uptake occurs as a result of the ingestion of sea water. The active absorption of sodium chloride, together with an isosmotic absorption of water, has been shown to occur in the gut of the larva of the alder fly, living in salt solution (*127*).

D. Extrarenal Secretion of Ions

In most animals, any difference between the loss of ions due to diffusion and the total entry due to diffusion and absorption is balanced by the removal of ions in the urine. An extrarenal secretion of ions has been demonstrated conclusively only in the marine teleosts (*51*).

The urine of the marine teleosts contains high concentrations of magnesium (Table III) and this is derived solely from magnesium in the intestine, resulting from the ingestion of large amounts of sea water (*51*). The chloride in the urine, however, is only sufficient to account for a small proportion of the chloride absorbed from this ingested sea water (14% in *Lophius* and only 5% in the sculpin) and hence most of it is excreted elsewhere. This has been firmly established, experimentally, by a study of the fate of salt solutions introduced into the intestine of eels adapted to fresh water (*51*). Magnesium and sulfate are invariably removed in the urine but large amounts of sodium, potassium, and chloride may be excreted extrarenally. The secretion of the monovalent ions takes place at the head end of the fish and the mechanism is presumably located in the gills.

In a preparation of the head region of the eel, severed just behind the heart and perfused with eel-Ringer through the heart and sea water through the gill chamber, Keys has shown that both water and chloride are transferred from the Ringer solution to the sea water (*7*). The water movement occurs in the direction of the osmotic gradient and does not take place if the outer solution is isosmotic with the perfusing fluid (*151*). The transport of chloride, however, takes place in spite of a large concentration gradient.

The rate at which chloride is secreted is determined by the concentration of the perfusing fluid. An increase in the osmotic pressure of the Ringer solution from $\Delta = 0.5$ to $\Delta = 0.7°$ increases the rate of secretion from about 4 mg./kg./hour to 30 mg./kg./hour (*7*). (see Fig. 4). The increase in osmotic pressure is an effective stimulus if it is brought about by the addition of sodium sulfate, glucose or sucrose, as well as sodium chloride, but not by sodium nitrate or urea, (*152*) so apparently the mechanism is concerned with the regulation of total concentration rather than with specific control of chloride balance.

A number of attempts have been made to identify special cells responsible for extrarenal secretion in fishes, although there is no *a priori* reason for supposing that such cells must exist. Large eosinophilic cells are found in considerable numbers in the gills of eels and these have been called "chloride-secreting cells" (*153*). Their presence in other marine teleosts is variable and they are also present in some freshwater fishes (*153*). The same cells have also been regarded as mucous cells in the process of development (*154*). In migrating fish, like the eel and *Fundulus*, the appearance and development of these cells may vary with the nature of the environment (*155, 156, 157*). In sea water the cells show greatest activity and this supports the theory that the cells are involved in the processes of osmoregulation.

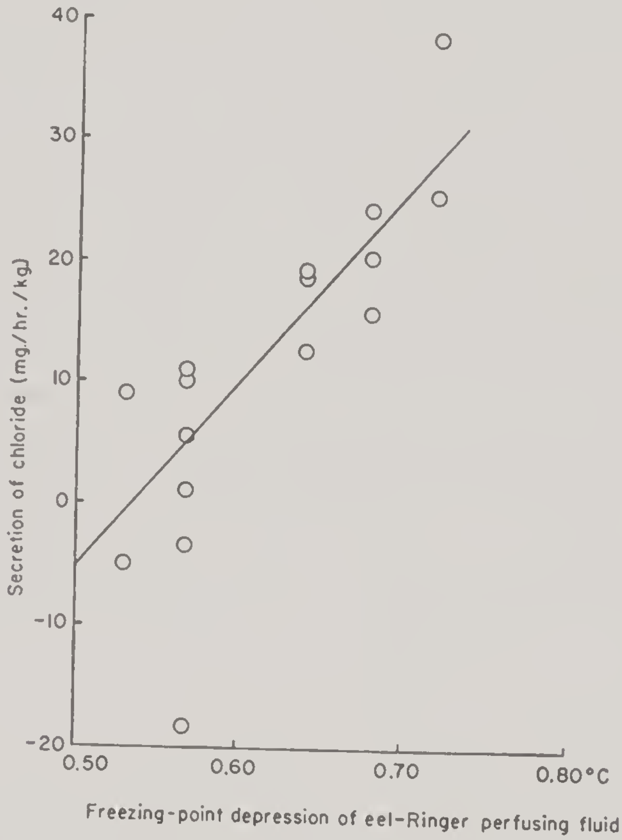

FIG. 4. The relation between the outward secretion of chloride by the perfused head of the eel and the total concentration (freezing-point depression) of the perfusing fluid. [Based on data from Keys (*7*).]

It has been suggested that extrarenal secretion of ions also occurs in the marine elasmobranchs (*46*). The magnesium concentration of the urine is often as high or higher than in the teleosts and the urine production rate is greater. By analogy with the teleosts, it might be supposed that much sea water is ingested and that the absorbed chloride is removed by extrarenal secretion. However, drinking apparently does not take place (or only to a very limited extent) (*46*) so there is no evidence for a large intake of chloride, and magnesium may enter by some other route. The urine contains considerable quantities of chloride (Table III) and it is possible that this is the only route for chloride removal.

IV. Osmoregulation in Cells and Tissues

In most higher animals, the cells and tissues of the organism are surrounded by the body fluid and have no direct contact with their outside environment. Important exceptions to this are found in the reproductive cells, which in many animals are liberated into the outside medium. In marine animals, these cells may well be isosmotic with sea water, as, for example, in sea urchin eggs (*158, 159*), or rapidly come into equilibrium, as in some fish eggs (*16*). Here no problems of osmoregulation exist. On the other hand, eggs of some teleosts like *Fundulus* (*160*), may remain hyposmotic to sea water and the eggs of freshwater animals are hyperosmotic to their environment. In the early stages of the development of these eggs, no special osmoregulatory mechanisms have been demonstrated; but the approach towards an equilibrium is delayed by the very low permeability of the bounding membranes. In the egg of *Fundulus* (*160*) and of the frog (*16*) the permeability to water is low, and the trout egg exhibits a complete impermeability to water for a short period during the early stages of its development (*161, 162*).

Some of the lower animals, which do not possess a body fluid, have penetrated into fresh water and their cells are in close contact with the environment at all times. This is most obvious in the unicellular protozoa, but the cells of some multicellular animals, like the freshwater sponges and coelenterates, are similarly placed. In these organisms a steady state exists between the cell and its freshwater environment, despite the differences in osmotic pressure and these differences must be maintained by the same processes which operate in the higher animals for the movement of water and ions.

In the freshwater protozoa, estimates of the total concentration of the cell contents, from direct measurements of vapor pressure in *Spirostomum* and *Pelomyxa* (*163, 164*) and indirect calculation for the peritrich ciliates (*165*), give values between 40 and 90 milliosmoles/liter. This is of the same order as the lowest level found in the coelomate animals (Table II).

Swelling and shrinking experiments and tracer studies (*164, 165*) have shown that the outer membranes of protozoans are permeable to water. The permeability to ions is not so well established. In *Spirostomum*, there is an interchange of radioactive sodium, potassium, and bromide between the cell and outer solution containing these ions (*44*) and ion permeability has been inferred from swelling experiments (*166*), although it is of a low order. As in the higher animals, osmoregulation is effected by processes which oppose both the osmotic uptake of water and the loss of salts due to diffusion. Very little is known of the mechanisms of ion absorption in the Protozoa, but the presence of the contractile vacuole has long been associated with the expulsion of water.

The contractile vacuole consists, in its simplest form, of a small fluid-filled vesicle which lies in the cytoplasm of the cell. This gradually increases in size and finally collapses, emptying its contents through a temporary pore in the cell surface. The vesicle then proceeds to fill again and the filling and emptying cycle is repeated at regular intervals. In simple vacuoles, such as are found in *Amoeba,* the increase in size may result from a coalition of a number of smaller vacuoles, but in the more complicated forms, as in *Paramecium,* fluid enters the vacuole from feeder canals which persist throughout the whole cycle. Contractile vacuoles are almost universally present in freshwater Protozoa and they may also be present in some marine and parasitic forms, especially among the ciliates.

In as much as the contractile vacuoles remove water from the organism, they are clearly involved in the maintenance of the steady state within the cell, and in some forms (possibly all) they constitute the major or only means for water removal. The amount of fluid expelled by the vacuole can be easily calculated from its maximum volume and the frequency at which it empties, and this amount has been found to correspond roughly with the amount of water entering by osmosis and through other causes, such as intake in the food vacuoles. In *Amoeba proteus,* for example, water permeability, calculated from changes in volume resulting from the application of hyperosmotic solutions, has been estimated at 0.026 μ^3/μ^2/atm./minute (*167*). In the same species, the rate of vacuolar secretion is between 0.036 and 0.09 μ^3/μ^2/minute (*168*) and, hence, for an osmotic pressure difference between the cell and outside of 1–2 atm., the fluid lost in the vacuole is roughly balanced by osmotic uptake. Similarly, in the ameboid, *Pelomyxa,* the water permeability, measured by means of the tracer technique, is of the same order (0.011 μ^3/μ^2/atm./minute), and again the osmotic uptake of water is about equal to the vacuolar output (*164*). In the ciliates, the surface membrane is probably more permeable to water. Kitching finds values between 0.05 and 0.1 μ^3/μ^2/atm./minute for a marine peritrich (*169*), and in a freshwater peritrich, *Carchesium aselli,* he calculates a value of 0.125–0.25 μ^3/μ^2/atm./minute by equating the rate of vacuolar output with the rate of water uptake (*165*).

In addition to water entering by osmosis through the cell surface, water taken up by other means, may take a significant contribution to the total water uptake. In *Carchesium*, some 8–20% of the vacuolar output may be due to water from food vacuoles (*165*) and, in actively-feeding *Paramecium* an even greater proportion may be involved (*170*). In this species the rate of vacuolar output is seen to vary with the rate of formation of food vacuoles (*171*) and there is an even more marked correlation between active feeding and vacuolar elimination in *Amoeba mira* (*172*). Perhaps the most striking example is the suctorian *Podophrya*, which, while feeding on *Colpidium* increases its vacuolar output to as much as 10 times the normal value. The decrease in the volume of the prey during feeding is roughly accounted for by the increase in the volume of the *Podophrya* and the additional vacuolar output (*173*). The fact that, in *Carchesium*, the vacuolar output is reduced to a very low level by the application of dilute cyanide solution, and that, as a result, a pronounced swelling occurs, is a further proof of the vital role of the contractile vacuole in water removal (*165*).

There is clear evidence that the total water content of the organism can be regulated by the variable activity of the vacuole (*166, 174*). Water intake can be controlled and varied in nonfeeding animals by reducing the osmotic gradient between the cell and its environment with external solutions containing a nonpenetrating solute. A reduction of the vacuolar output corresponding to a reduction in the osmotic gradient has been demonstrated in many freshwater Protozoa (*166*) and particularly clearly in the case of *Carchesium* and *Podophrya* (*165, 175, 176*). *Podophrya* shows a linear relation between the concentration of an external sucrose solution and the rate of vacuolar output (see Fig. 5) (*176*). At a sucrose concentration of 0.04–0.05 *M* the vacuolar output ceases and this solution is presumably isosmotic with the cell contents. In *Carchesium*, a similar relation is found, except that the regulation is not so exact and, in an isosmotic solution, the vacuole still discharges a small amount of fluid. The swelling, which results from the application of cyanide to the organism in fresh water, can be prevented by the application of an isosmotic solution (*175*).

The control of vacuolar output is not always so precise as in *Carchesium* and *Podophrya*. In the marine peritrichs, a reduction of the concentration of their environment from 100% to 75% sea water has little effect on the rate of fluid production by the vacuole and an efficient regulation is only established at much lower concentrations (*177, 178*). Also in the freshwater rhizopods, it has yet to be conclusively demonstrated that regulation of vacuolar output occurs in response to changed osmotic gradients, and the results are conflicting (*172, 174, 179*).

In *Carchesium* and *Podophrya*, an increase in the osmotic gradient, achieved by treatment of the organisms in glycol, glycerol, or 10% sea-water solutions and subsequent return to fresh water, leads to an increase in vacuolar output (*175, 176*). Temperature also effects the output and this is probably related to its effects on the permeability of the membrane to water and, hence, on the water intake (*175*). The variations in vacuolar output, resulting from changes in osmotic gradient, water intake with food, and temperature effects, all show how closely the output is geared to water intake, but they do not reveal the factor which regulates the vacuolar output rate. It is unlikely that this factor is the osmotic pressure of the cell contents, since the vacuolar output may increase when the latter is normal or, even, higher than normal. Several facts suggest that body volume may be the operative factor (*174, 176*), though, if this is so, the organism must be sensitive to very small changes. Kitching calculates that, in *Podophrya*, a 1.5% change in the body volume would be sufficient to bring about a complete cessation of the vacuolar activity (*176*). Even if this should prove to be the primary cause of changes in the rate of vacuolar secretion, the means by which a change in body volume could influence the activity of the vacuole is completely unknown.

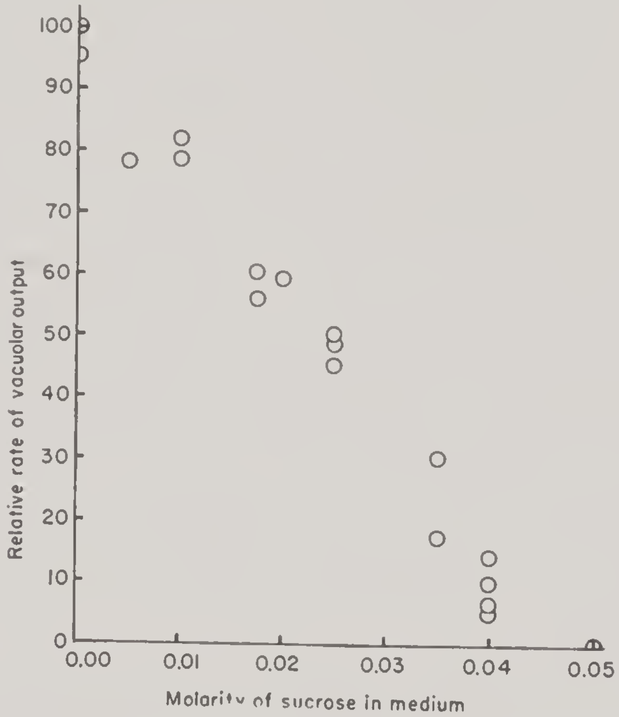

FIG. 5. The relation between the rate of output of fluid by the contractile vacuole of *Podophrya* and the concentration of sucrose in the medium. The points represent the rate of vacuolar output in the sucrose solutions expressed as a mean of the percentages of the original rate and of the final rate in tap water. [Based on data from Kitching (*177*).]

Nothing is known of the composition of the vacuolar fluid. Indirect evidence suggests that it consists of pure water or a very dilute solution (*174*) and, if this is so, then the vacuolar membrane must be the site of osmotic work—a view which receives support from the fact that cyanide and other metabolic inhibitors greatly reduce the activity of the vacuole. Although the direct secretion of very dilute solutions is extremely rare in animals, it must be remembered that, in the freshwater Protozoa, the gradient against which water would be secreted is quite small.

Very little is known of the osmoregulatory mechanisms in other invertebrates which are without body fluids. The cells of the freshwater sponge, *Spongilla*, are probably hyperosmotic to their environment (*43*) and some, at least, of the freshwater sponges have contractile vacuoles. A few coelenterates are found in fresh water. In *Hydra*, the cells are rather highly permeable to water and the osmotic pressure of the cell contents is distinctly higher than that of their environment (*42*). There are no visible structures which can be associated with water removal and the means by which this is achieved is not known. In *Pelmatohydra*, the animal, as a whole, is permeable to sodium, potassium, and bromide ions and these ions may be present in concentrations greater than that of the outside fluid (*42*). Presumably mechanisms for both ion and water transport are located in the cell membranes, but which of the ions are actively transferred is not yet known (*42, 179*).

It has generally been assumed that the tissue cells of all coelomate animals are isosmotic with the extracellular fluids. The few measurements on invertebrates, confined to direct measurements of intracellular concentration of muscle cells (*16, 159*), have confirmed this view. Similarly, among the vertebrates, measurements of the vapor pressure of frog muscle are also in agreement (*180*).

In mammalian cells, however, the concept of cell isosmoticity has been called into question. The liver and kidney of the rat swell, when isolated, with the uptake of water (*181*). The same phenomenon is observed in slices of these organs, isolated in Ringer solution, when their respiration is inhibited by cyanide, dinitrophenol, or by cooling (*182, 183*). In both cases, swelling may be prevented by the use of a Ringer solution of about twice the normal concentration. In the case of the tissue slices, the cell volume is returned almost to normal on removal of the metabolic inhibitor.

It has been suggested that the cells are not in simple osmotic equilibrium with the body fluid and that their volume is actively regulated (*181*). It is very striking that, in many respects, the behavior of many mammalian cells is remarkably similar to that of the freshwater Protozoa, although, of course, no visible vacuoles are present. Robinson believes that water is pumped outwards across the cell membrane to maintain the steady state—a process requiring energy and which would be stopped by the metabolic inhibitors.

The theory of active water expulsion by the mammalian cell has not been widely accepted. The liver and kidney cells, like most other cells, have an ionic composition very different from that of the extracellular fluids (*184, 185, 186*) and active processes maintain high potassium and low sodium concentrations. Interruption of metabolism or respiration leads to a loss of potassium and an increase in the sodium concentration; swelling may result from the isosmotic uptake of water which follows the penetration of sodium (and chloride) into the cell (*187–188a*). The recovery of the cell volume in well-oxygenated conditions, could be explained by the reactivation of the sodium-extrusion mechanism and, hence, the removal of sodium chloride from the cell, together with water (*189*) although the active secretion of water still remains a possibility (*190*).

It is always difficult to separate water movements, *per se,* from those which result from the simultaneous movement of ions. A more direct proof of the existence of osmoregulatory mechanisms in mammalian cells would come from an unequivocal demonstration that the cell contents are hyperosmotic to the extracellular fluids. Unfortunately, technical difficulties make the interpretation of measurements on cell contents somewhat ambiguous, and recent careful studies on the freezing-point depression of mammalian tissues have led to conflicting results (*189, 191–196*). At present, the balance of evidence does not favor the occurrence of hyperosmotic cells, but the possibility cannot yet be completely dismissed.

V. Some General Conclusions

All freshwater animals, and some forms from brackish water, maintain an internal osmotic pressure greater than that of their environment. Among the animals with body fluids, the problems of the maintenance of this steady state have been overcome in a wide variety of different ways. Yet, despite this, the processes of hyperosmotic regulation, which involve the movements of water and salts in a direction opposite to that of the net movements due to diffusion, are achieved by a few well-defined mechanisms. In the great majority of cases, these mechanisms make use of existing organs, such as the excretory organs or gills, which have been developed for other purposes. The same type of osmoregulatory mechanisms have evolved independently many times.

The movement of water and salts, against the diffusion gradient are processes requiring the expenditure of energy by the organism. Since the permeability of the outer membranes of the organism to water and salts determine the extent of the net movements due to diffusion, it is clear that the total energy requirements for osmoregulation depend on surface permeability. In freshwater animals, the reduction in permeability, and the consequent reduction in the demands on the metabolism for osmoregulatory purposes, is undoubtedly one of the most important adaptations to life in this environment. Potts has also stressed the importance of the production of a dilute urine (*197*) but this only becomes valuable when the salt permeability of the surface is low (*198*). This is not the case, for example, in *Eriocheir*, where, in fresh water, the greater part of the salt loss occurs through the surface, despite the production of an isosmotic urine (*9*).

The uptake of ions against the diffusion gradient is effected by ion-transporting mechanisms located in the surface membranes—often making use of the respiratory epithelium. In a few cases, like the eel and some freshwater insect larvae, the permeability of the surface to salts is so low that no special uptake mechanisms are present and such salt intake, as does occur, must take place through the gut. The membranes, responsible for ion transport, are often composed of a single layer of homogeneous cells without special morphological features. Their property of transporting ions probably represents an exploitation of existing transporting mechanisms, located within the membranes of the cells themselves and originally responsible for the maintenance of intracellular composition. In this respect it is worth noting that in the amphibian skin and probably in other uptake sites, most work is involved in the transport of sodium ions and it is these ions which are actively secreted from many cells.

The removal of water is not brought about by the active secretion of water molecules, except, perhaps in the Protozoa and the cells of certain other animals. In animals with body fluids, water is generally separated, initially, by a process, such as filtration which requires a minimum of work. The water is accompanied by an isosmotic concentration of salts; thus, although the process is economical as regards the movement of water molecules themselves, energy must be utilized in the recovery of salts by reabsorption in the distal part of the excretory tubule, as in freshwater animals like the frog, fish, and crayfish, or by the additional uptake of ions at the surface, as in forms like *Eriocheir*, which produce an isosmotic urine. Where urine is produced by secretion rather than filtration, as in aquatic insects, again little work is done in transporting water molecules, but energy is used in the secretion of ions and their subsequent reabsorption—a striking example of the apparent inability of cellular membranes to secrete water against a large osmotic gradient.

The total energy required for the maintenance of a hyperosmotic state in higher animals, is utilized almost exclusively for the active transport of ions. The energy is consumed either entirely at the surface or shared between the surface and the excretory organ tubule. Where the work is shared, the proportioning of work between surface membrane and excretory organ depends, both on the relative permeability of the body surface to water and to salts, and on the efficiency of the excretory tubule cells in removing ions from the urine.

For the maintenance of a hypo-osmotic state, the processes are reversed—water must be taken up and salts secreted against the diffusion gradient. In the marine teleosts the mechanisms by which this is achieved is clear. Water is absorbed, isosmotically with salt, from the intestine; but the bulk of the osmoregulatory work is done in the active secretion of the monovalent ions through the gills. This mechanism removes ions taken up from the intestine and also those which enter through the body surface by diffusion. A small proportion of the total work is also involved in the secretion of the divalent ions in the urine.

In other hypo-osmotic forms, the mechanisms are still not clearly defined. In some Crustacea, it is possible that salt secretion occurs only by way of the isosmotic urine and that the osmotic work is done solely by the active uptake of water; but in *Artemia salina* extrarenal secretion may make an important contribution to the osmoregulatory mechanisms (*199*). In the saline insect larvae, like *Aedes detritus*, also, the mechanisms are still uncertain. Drinking of salt water probably occurs and a hyperosmotic urine is produced, but whether this is achieved by the active reabsorption of water or the secretion of salts is not known.

The marine elsamobranchs form a very interesting intermediate stage between hypo- and hyperosmotic regulation in fish. Because of the high concentration of urea in the blood, they are slightly hyperosmotic to the surrounding water, although the salt concentration is low. The processes of osmoregulation involve both the removal of water and salts, since their diffusion gradients are, in these animals, in the same direction. Both these processes may well be effected by the excretion of an isosmotic urine and the total osmoregulatory work would be very small, involving only the secretion of the divalent ions and the reabsorption of urea in the kidney tubules. The possibility of additional extrarenal secretion in the elasmobranchs has yet to be substantiated.

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CHAPTER 10

Nerve Conduction and Electrical Discharge

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I. Introduction

During recent years, remarkable progress of our knowledge in the field of cellular permeability has been achieved. It would now seem to be well established that the free energy accumulated in cells as energy-rich phosphates is, among other things, utilized to achieve the unequal distribution of ions between a cell and its surroundings and to maintain the structural organization responsible for the various permeability characters of living membranes. As a result, free energy is stored in the form of ionic gradients across cell membranes. Active transport of ions seems to be an essential feature of all living cells since we do not observe striking differences whether we consider a monocellular organism or a vertebrate. In multicellular organisms however, we find tissues where active transport appears to be the only function and the only "purpose" of the differentiation: the kidney, the gastric mucosa, the various glands are typical examples. There are also examples where the importance of active transport is not immediately recognized, despite the fact that it is an integral part of a function. It is only very recently that the importance of the unequal distribution of ions between a nerve and its surroundings has been recognized. In an excitable structure the ionic gradients are indeed responsible for the local electric circuit characteristic of the action potential. Electrical activity appears therefore to be the result of the utilization for a specific requirement of a wide-spread property of living matter. Through various types of morphological adaptations this electric current is then used to transmit messages between distant points of an organism, to integrate functions, to provide for mechanisms of defense and attack, etc.

The reader will find in the following pages the facts upon which this concept is based.

II. Ionic Basis of Bioelectric Potentials

A. IONIC DISTRIBUTION BETWEEN CELLS AND THEIR SURROUNDINGS

Biologists have known for a long time that the ionic composition of the interior of most cells is quite different from that of the extracellular fluid (*1, 2*). It is however very difficult to know the exact composition of intracellular fluid owing to the very great difficulty of obtaining accurate measurements of ion concentration in a tissue in which a large proportion of the ion may be in the extracellular space. Only in a few cases can a direct estimation be made. In the squid giant axon for instance, samples of axoplasm can be obtained by extruding the content of the axon. Axoplasm obtained in this way provides an excellent material for chemical analysis (*3–5*). When we are dealing with cells other than the giant axon we have to rely on indirect methods generally based on (*a*) the study of the kinetics of the washing out of the ion under investigation (*6–8*), and (*b*) some assumptions concerning the composition of the extracellular space. The biological material is generally considered to be a system formed by a number of compartments in series or in parallel. The mathematical treatment of the cases most often encountered in biology can be found elsewhere (*8–14*). The compartment representing the extracellular space is generally assumed to have the same composition as the plasma. The sheaths surrounding the axons might, in vertebrates, be an extension of the sheaths surrounding the brain. The extracellular fluid would in this case have the composition of the cerebrospinal fluid, which does not differ much from that of the plasma. But in some insects the hemolymph can be exceptionally rich in K and poor in Na (*15*), the reverse of what is to be expected (see below). The assumption that in this case the extra-axonal fluid could have a different composition from that of the plasma is enhanced by the fact that if hemolymph is injected inside the sheaths of an insect nerve, nerve conduction is blocked (*16*).

TABLE I

CONCENTRATIONS OF SODIUM AND POTASSIUM IN EXCITABLE CELLS AND BODY FLUIDS[a,b]

	Tissue		Fluid	
	K	Na	K	Na
Muscle				
Grasshopper	128	44	18	64
Cockroach	112	45	17	107
Squid	114	54	16	350
Thyone	169	170	10	450
Frog	85	24	3	104
Cat	113	21	6	150
Rat	158	18	4.6	138
Nerve				
Crab	158	168	10	450
Squid (axoplasm)	375	72	16	350
Frog	48	62	3	104
Electroplax				
Electric eel (millimolars)	152	20	5.8	185

[a] In millimoles per kilogram wet weight, except when otherwise stated.

[b] After Steinbach (*17*), except for the data for the rat from Creese (*18*) and for the electric eel from Schoffeniels (*19*).

Table I gives a few data for K and Na concentrations. It will be seen that the intracellular fluid contains a high concentration of potassium and a low concentration of sodium (and chloride). This is the reverse of the situation in the plasma where sodium and chloride are the dominant ions. The mobility and diffusion coefficient of labeled potassium in the axoplasm of the squid are about the same as in a free solution (*20, 21*). This and other results (*22*) seem to indicate that potassium ions are probably in the free state inside the fiber and not bound to large molecules (proteins, for instance).

The excess of K inside the fiber is balanced by organic anions, among which aspartate, glutamate, and phosphate esters are the most important (*23, 24*). In the case of the axoplasm of the squid, isethionate is quantitatively the most important (*25, 26*).

As shown in a preceeding chapter (cf. Andersen and Ussing, Vol. II, Chapter 7), the unequal distribution of ions is due to a mechanism of active transport of Na (perhaps by a forced exchange of Na for K) and to the specific permeability of the membrane to certain ions.

B. POTENTIAL DIFFERENCE ACROSS EXCITABLE MEMBRANES AT REST

There is a structure outside the axoplasm which acts as a barrier, slowing down the movements of ions between the cell interior and extracellular fluid. The external fluid is thus prevented from mixing rapidly with the internal fluid. This barrier is generally referred to as the cell membrane; it is thought to be the site of the ionic regulation and would appear to be involved in the mechanism whereby the action potential is produced. It has high electrical resistance (a few thousand ohm cm.^{2}) corresponding to a small permeability to ions, while the electrical resistance of the cell interior as well as that of the extracellular fluid is approximately 30 ohm cm.^{2} (*27, 28*). There is a potential difference between the two phases separated by the membrane. The

TABLE II

MAGNITUDE OF BIOELECTRIC POTENTIALS RECORDED IN VARIOUS ANIMALS[a]

	Potential difference		References
	At rest	During activity	
Loligo forbesi	−50	+90	Hodgkin and Huxley (*29*)
Loligo pealii	−51	+104	Curtis and Cole (*30*)
Sepia officinalis	−62	+120	Weidmann (*31*)
Homarus vulgaris	−62	+106	Hodgkin and Huxley (*32*)
Carcinus maenas	−71 − −96	+116 − +153	Hodgkin and Huxley (*32*)
Electrophorus electricus L. (electroplax)	−80 − −90	+125 − +144	Schoffeniels (*19*)
Rana esculenta	−71	+116	Huxley and Stämpfli (*33*)
Rana temporaria (muscle)	−88	+119	Nastuk and Hodgkin (*34*)
Rana pipiens (cardiac fiber)	−50 − −90	+65 − +115	Woodbury *et al.* (*35*)
Canis familiaris (Purkinje fiber)	−90	+121	Draper and Weidmann (*36*)
Capra hircus (Purkinje fiber)	−94	+135	Draper and Weidmann (*36*)

[a] The figures are given for nerve fibers except where otherwise stated. Potential difference in millivolts; the sign refers to the inside of the cell.

cell interior is generally negative with respect to the cell exterior by 50 to 90 mv. (Table II). This potential difference is generally what is expected from the unequal distribution of K providing that (*a*) the membrane is more permeable to K than to other ionic species, and (*b*) the activity coefficients of K inside and outside the cell are equal. It now seems to be well established that this potential difference is an electrochemical potential. The main line of argument is based on the observation that there is a certain proportionality between the potential difference observed and the logarithm of the outside concentration of K (*30, 37–40*). According to the Nernst equation[*] there should be a change of 58-mv. for a 10-fold change in the outside K concentration (at 20°), over any range of concentration. But this is not always the case and in the lower range of concentration, the discrepancy between theoretical and observed values is very large (Fig. 1). This has been explained by the fact that the membrane is not only permeable to K but also has a finite permeability to other ions, especially Na and Cl (*40a*).

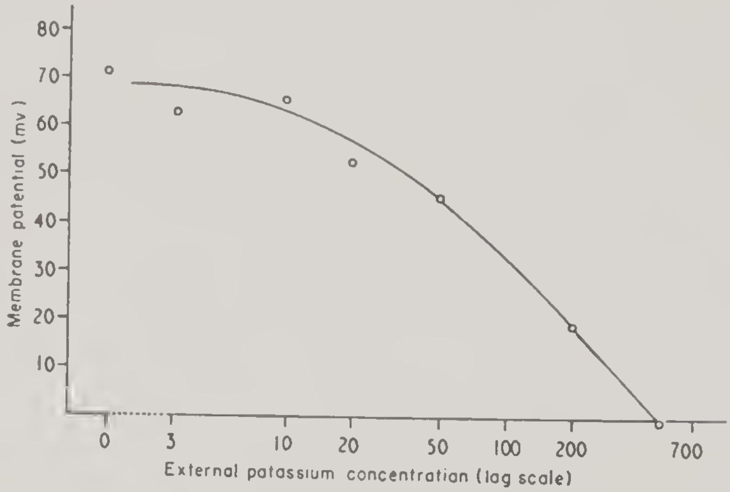

FIG. 1. Relation between external potassium concentration (m*M*) and the potential difference of *Sepia* axons at rest [after Hodgkin and Keynes (*39*)].

The Nernst formula is therefore inadequate to relate potential difference and ionic distribution. Hodgkin and Katz (*41*) have derived from the constant field equation of Goldman (*42*) an expression taking

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[*] The simple formula commonly associated with Nernst's name has the following form:

$$E = \frac{RT}{zF} \ln \frac{c_{\text{out}}}{c_{\text{in}}}$$

where E is the electromotive force, R the gas constant, T the absolute temperature, F the Faraday constant, z the valence of the ion, c_{in} and c_{out} the concentrations of the ion in the two phases.

into account the relative permeability of the ionic species which are numerically the most important. The potential difference E, across a membrane is then given by:

$$E = \frac{RT}{zF} \ln \frac{P_K(K)_{out} + P_{Na}(Na)_{out} + P_{Cl}(Cl)_{in}}{P_K(K)_{in} + P_{Na}(Na)_{in} + P_{Cl}(Cl)_{out}} \qquad (1)$$

where P_K, P_{Na}, and P_{Cl} are the permeability coefficients of K, Na, and Cl. The subindexes "in" and "out" refer to the inside and outside of the cell respectively.

It is of course assumed in this formulation that the structure of the membrane is stable and that changes in the ionic environment in no way affect the permeability characteristics of the membrane. This obviously is a rather simplified assumption and it seems that a formulation such as Eq. 1 can only be applied with extreme caution to living systems. It is a well known fact that an increase in the outside K concentration results in a decrease in the resistance of a conducting membrane while inverse changes take place with Ca (*43, 44*). Modifications of resistance are of course dependent on some structural changes and one should expect that some kind of interrelation between the various ions is actually involved in determining the permeability characteristics of a living membrane. In fact it has been shown that the potential difference measured across the innervated membrane of the electroplax of *Electrophorus electricus* L. is a function of both K and Ca concentration in the bathing fluid. At high Ca concentration (6 m*M*) the potential difference across the innervated membrane is insensitive to the concentration of K in the bathing fluid, while the reverse is true at low Ca concentration (1 m*M*) (*19, 45*). It could be that at high Ca concentration the potential difference is mainly due to the unequal distribution of Cl ions.

C. The Action Potential

The electrical activity of excitable structures is usually explored by applying cathodal stimulation to the surface of the membrane. With currents of small strength, a negative wave appears which is proportional to the strength of the stimulus and which declines exponentially as it spreads along the surface of the excitable membrane (*passive depolarization*). The decremental transmission of such a wave of depolarization is known as electrotonic spread and is analogous to the propagation of electric pulses in a cable which is very leaky and has a high electrical capacity (*46, 47*). This type of response depends on the cable-like properties of the nerve fiber with its highly resistant membrane and relatively good conducting core. By this mode of transmission, an electric signal can only spread over distances of a few millimeters.

If the strength of the stimulus is increased, the depolarization wave gives rise to a response of the excitable membrane, i.e., electrical energy is released in the form of a superimposed depolarization wave (*active depolarization*). This is the *graded response* (proportional to the strength of the stimulus applied). The graded response is also called *local response* because, like the electrotonic spread, it is conducted decrementally over a short distance.

If the strength of the stimulus is further increased, the potential difference which normally exists across the membrane is eventually reduced to a critical level (threshold). At this point the response of the membrane is maximal, does not depend on the strength of the stimulus applied (*all-or-none response*), and is self-reinforcing and thus propagated without decrement along the membrane; this is the *spike* or *action potential.*

Figure 2 gives some quantities in the case of the single isolated electroplax of *Electrophorus electricus* L. (electric eel). It has indeed been shown that with a cell of this sort it is possible to elicit an action potential identical to that of a nerve fiber (*48*). The resting potential is about 90 mv., the inside of the cell being negative with respect to the outside. Application of an electrical stimulus rapidly brings the potential difference to about 70 mv. At this point the cell is activated and an action potential is produced. The amplitude of the spike is about 140 mv. This means that at the peak of activity, the interior of the cell becomes 50 mv. positive. The threshold depolarization (i.e., the reduction of the resting potential needed to activate the cell) is 20 mv. Thus during activity the membrane potential reverses sign, the total change in potential being several times greater than that initially required to ex-

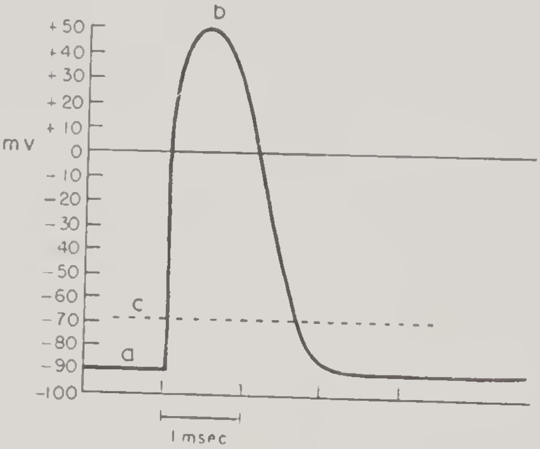

FIG. 2. Action potential recorded with an intracellular electrode from a single isolated electroplax of *Electrophorus electricus* L. *a*, resting potential; *b*, maximum potential difference during activity; *c*, threshold level of activation (from unpublished recordings by Schoffeniels).

cite. This observation was first made on the giant axon of the squid (*43–50*).

An important advance in our understanding of the mechanism responsible for the action potential was the demonstration by Cole and Curtis (*49*) that there is a breakdown in membrane resistance as postulated long ago by Bernstein (*37*). According to the theory he developed as early as 1902, axons are surrounded by a semipermeable membrane, selectively permeable to K. When a stimulus activates the membrane, the permeability is greatly increased for all ions. The active part becomes depolarized, a small electric current is then generated which activates the adjacent points, and the same process takes place there.

As shown above, Bernstein's theory is still the basis of our concepts. Only one modification has become necessary: the action potential is not a simple depolarization but a reversal of polarity. During activity the permeability to both Na and K increases, with the net result that each impulse is associated with an increased influx of Na and an increased outflux of K (*50, 51*). Table III gives some results of studies on ion movement associated with the generation of electrical activity in various excitable structures.

TABLE III

NET MOVEMENT OF CATIONS DURING ELECTRICAL ACTIVITY IN NERVE FIBERS OF VARIOUS ANIMALS AND IN THE ELECTROPLAX OF ELECTRIC EEL[a]

	K loss	Na gain	References
Carcinus maenas	1.7	—	Hodgkin and Huxley (*52*)
Sepia officinalis	4.3	3.7	Keynes (*53*)
Loligo forbesi	3.0	3.5	Keynes and Lewis (*5*)
Loligo pealii	—	4.5	Rothenberg (*54*)
Electrophorus electricus L.	—	0.9	Schoffeniels (*19*)

[a] Flux in micromicromoles per square centimeter per impulse.

A simple way of explaining the action potential is by assuming that, when the membrane is activated, the permeability increases in such a way that the membrane initially becomes permeable to Na. In support of this idea it can be shown that, in the absence of Na or Li, electrical activity disappears. This observation was made by Overton (*55*) who suggested that conduction could be associated with an exchange of extracellular Na for intracellular K. The availability of radioactive isotopes made it possible to measure the ion movements during activity on a quantitative basis and it is now well established, mainly through the work of Hodgkin and his collaborators (*51*), that at the peak of the spike the potential measured approaches the value of the equilibrium potential for Na. The sequence of events is as follows. The activation of the membrane first consists of a sudden increase of the membrane permeability to Na followed by an increase in K permeability. The rising phase of the spike coincides with the influx of Na while the falling phase corresponds to the outward movement of K. Consequently the amplitude of the spike must be directly related to the outside Na concentration. In fact it is found that most of the excitable tissues behave like a sodium electrode at the peak of activity (*34, 56*).

An interesting observation, first reported by Lorente de Nó (*57*), is that frog nerve can still produce action potentials in a Na-free medium if tetrabutylammonium ions or other ammonium derivatives are added. This observation has been extended to other excitable tissues (*58–60*) and raises the important question: What ion is carrying the current under these experimental conditions? It has been suggested (*59*) that the ammonium derivatives used as a substitute for Na could play that role. This explanation is unlikely however, since one still gets an action potential whether the ammonium derivatives are applied intracellularly or extracellularly (*58*). One must then assume that other extracellular cations (maybe calcium) or other intracellular anions (amino acids?) are responsible for the observed electrical activity.

To summarize briefly, the unequal distribution of ions between a cell and its surroundings is due to a mechanism of active transport. Owing to the permeability characteristics of the membrane surrounding a cell, a potential difference is established. What differentiates an excitable membrane from any other membrane is the possession of a mechanism enabling the membrane to change, suddenly and for a very short period of time, its permeability characteristics. The energy stored as ionic gradients can then be used as the source of the electromotive force (E.M.F.) known as action potential.

III. Nature of the Permeability Change

Whereas there is little disagreement (*47, 61*) about the nature of the spike (permeability change, ionic movements, electrochemical potential), there are strongly opposing views concerning the nature of the mechanism underlying the permeability change, the primary event in the action potential. This is the only manifestation of living cells for which at present a purely physical process is generally offered as an explanation by leading neurophysiologists (*62*). On the other hand, some authors maintain the view that chemical reactions must control the permeability change (*63–66*). The measurement of heat production during electrical activity and the temperature coefficient (Q_{10}) of this process are topical sources of information in answering the question of whether purely physical processes could account for the generation of the spike.

There are relatively few data in the literature concerning the temperature coefficient of the spike and they are generally not considered as relevant to the problem of the nature of the phenomenon involved (*67*). However, since the spike is directly related to the permeability change, the duration of the spike must be a good measure of this change and must be pertinent to the question of whether or not chemical reactions are involved in the process.

Recently, using the isolated single electroplax of the electric organ of *Electrophorus electricus* L., the Q_{10} and the energy of activation of the electrical activity have been evaluated over a wide range of temperatures (*68*). The duration of (*a*) the action potential, (*b*) the latency period, and (*c*) the postsynaptic potential has been studied. The duration of all three phenomena decreases with a rise of temperature, whereas the amplitude of the spike is little affected. Figure 3 shows the results obtained with the spike elicited by direct stimulation.

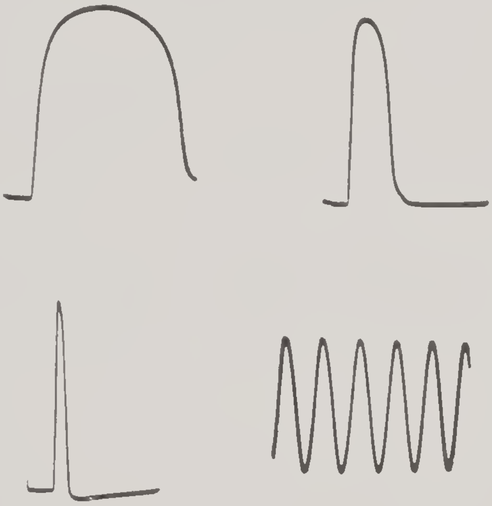

FIG. 3. Action potential elicited by direct stimulation and recorded from a single isolated electroplax (*Electrophorus electricus* L.) at various temperatures. From upper left to lower right: 12°; 24°; 39°; calibration 50 mv., 2 msec. [Schoffeniels (*68*)].

When the logarithm of the reciprocal of the half-width of the action potential is plotted against the reciprocal of the temperature according to Arrhenius, a straight line is obtained (Fig. 4). This enables us to assign an energy of activation to the rate-controlling step in these processes. The action potential elicited by direct stimulation has a Q_{10} of approximately 3.6 and an energy of activation of 21,000 cal./mole. The Q_{10}'s of the latency period and of the postsynaptic potential are very close to 2.6, and the energy of activation is around 16,000 cal./mole.

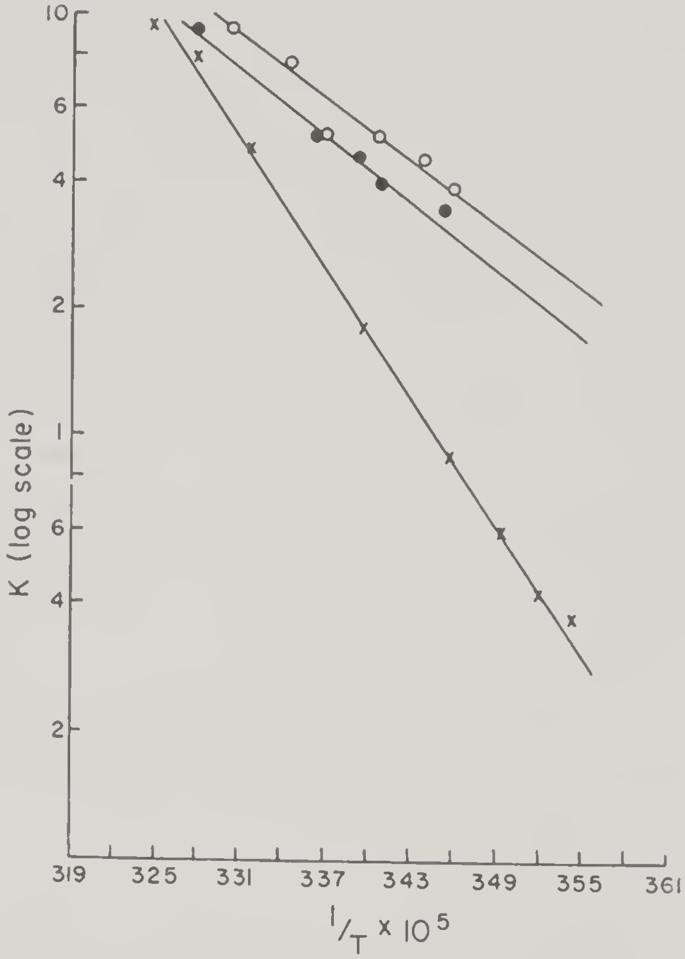

FIG. 4. Logarithm of the half-width (K) of the action potential (×) and of the postsynaptic potential (○) plotted against the reciprocal of the absolute temperature T according to Arrhenius. ●, reciprocal of the duration of the latency period against the reciprocal of T.

$$\ln K = -\frac{E}{RT} + \text{constant}$$

Although it is true that some chemical reactions may have low Q_{10}'s, there is not a single physical process known so far to have a Q_{10} above 1.4. Thus the data support the conclusion that the rate-limiting factor of the three phenomena is dependent on chemical reactions. This is consistent with recent observations on the initial heat production in nerve fiber (*69*) and in electroplaxes of *Torpedo* (*70*). Abbott *et al.* (*69*) have indeed succeeded in recording the initial heat production of a nonmedulated nerve (*Maja*). They were able to separate the initial heat change into two phases: a positive heat production, averaging in a single impulse 9 × 10^{-6} cal. per gram nerve (at 0°). This phase is rapid (approx. 60 msec.). In the second phase, lasting about 300 msec., heat is absorbed averaging 7 × 10^{-6} cal. per gram nerve. Previously, only the net heat, i.e., the difference between the two processes, was recorded. Among some explanations for the type of heat production observed, the authors discussed the possibility that, with the membrane behaving as a condenser,* the heat produced would come from the discharge and recharge of the membrane. This hypothesis is extremely attractive because, as shown by Abbott and collaborators (*69*), the positive and negative heats recorded are of the right order of size. However, the drawback to the "condenser theory" is that the time relation seems to be wrong. The negative heat lasts for a time of between 100 and 300 msec.; if the recharging of the condenser is to account for it, the duration of the action potential would have to be of that order of magnitude. The heat of ionic mixing (intracellular K with extracellular Na) would also be too small to account for the initial positive heat measured. A fact in favor of the theory that the origin of the heat is due to a chemical reaction is the absence of any marked effect of temperature on the net heat; for if the chemical processes underlying the permeability change are the same at various temperatures, though occurring more rapidly at higher temperature, the heat production should be the same and independent of the temperature.

We further consider that the high values of the energy of activation plus the fact that the initial heat can be separated into two phases provide support for those theories which postulate chemical processes to be responsible for the specific changes in permeability which enable the excitable membrane to generate an electric current.

IV. The Permeability Change

Although the study of the influence of temperature on the electrical activity or the measurement of heat production accompanying the spike gives some general type of information concerning the nature of the process, these results do not give any idea of the molecular forces involved in the permeability change. This brings us to the question: What chemical reactions are responsible for the transitory change in permeability by which the potential source of energy, the ionic concentration gradient, becomes the effective source of an electromotive force?

Calcium is thought by many authors to play a major role in the generation of the spike (*62*). Although the importance of Ca in permeability studies is generally acknowledged, its exact function remains nevertheless obscure and it has not yet been fully understood (*71*).

Abood and Abui-Haj (*72*) suggest that the permeability change responsible for the spike is due to a proper balance between depolymerization and repolymerization of hyaluronic acid. A. von Muralt (*73*) emphasized the role of thiamine in the excitability process and Ungar *et al.* (*74, 75*) have shown that during stimulation, changes in protein configuration occur by the breaking of hydrogen bonds. These workers suggest a possible relationship between the changes observed and the breakdown of resistance in the excitable membrane. On the other hand Tobias (*76*) stresses the importance of the lipid fraction of the membrane in the generation of an action potential.

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* The equivalent electrical circuit of a living membrane is that of resistance in parallel with capacity.

The only coherent theory which attempts to picture the permeability change at a molecular level has been developed by Nachmansohn. Despite its imperfections, it is the most fruitful working hypothesis as yet proposed, one which attempts to integrate the vast number of observations accumulated not only by pharmacologists but also by neurophysiologists.

The most important facts pertinent to this theory will therefore be summarized in the following paragraphs. More detailed accounts may be found elsewhere (*64*).

At the beginning of the present century, two substances were recognized as being important for nerve function: adrenaline (*77*) and acetylcholine (*78*). On the basis of pharmacological studies and by analogy with the remarkable simultaneous progress in the field of endocrinology, these substances have been thought to play a role as mediators in the transmission of nerve impulses across synaptic junctions. In the case of acetylcholine three essential facts form the basis of the mediator theory: (*a*) the action of acetylcholine at synaptic junctions; (*b*) the appearance of acetylcholine in the perfusion fluid of the synapse following nerve stimulation; and (*c*) the electrical inexcitability of the postsynaptic membranes. Whereas the action of acetylcholine might have been considered to be of pharmacological interest only, the two other observations clearly suggested a physiological role for this ester. Indeed it was later shown that not only acetylcholine but also choline acetylase and acetylcholinesterase were present at synapses as well as in many excitable membranes. Moreover it became apparent that the tertiary amines and the quaternary ammonium derivatives, known to interfere *in vitro* with the acetylcholine-cholinesterase system, were able to affect electrical activity. These and other observations led Nachmansohn to propose, as an extension of the concept of neurohumoral transmission, a theory based on the essentiality of acetylcholine in electrical activity. In the cycle he proposes (Fig. 5), acetylcholine is released by current flow (possibly hydronium ions), or any other excitatory agent, from its bound form (combined with the storage protein, S). The free ester combines with a structural element of the membrane, the receptor *R*. The receptor changes its configuration (dotted line). As a result, the permeability increases. This is the trigger action by which the ionic concentration gradient becomes effective. The acetylcholine-receptor complex is in dynamic equilibrium with the ester and the receptor. The free acetylcholine is then accessible to hydrolysis by the esterase *E*. This permits the receptor to return to its original configuration. As a consequence, the permeability decreases. Acetylcholine is then resynthesized by choline acetylase.

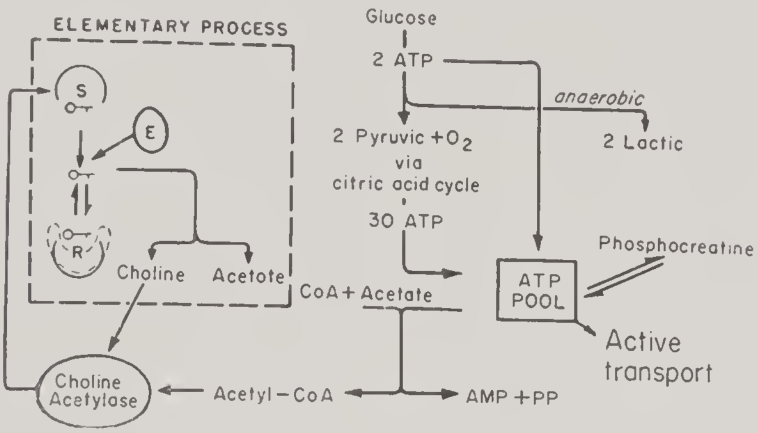

FIG. 5. Nachmansohn's cycle. Primary event responsible for the permeability change (elementary process) and sequence of energy transformations associated with the generation of an action potential [Nachmansohn (*64*)].

In his cycle, Nachmansohn postulates the existence of four proteins (or conjugated proteins):

(*a*) the storage protein,
(*b*) the receptor protein,
(*c*) the esterase,
(*d*) the choline acetylase.

At present, only two of the four proteins postulated have been isolated in solution: the esterase and the choline acetylase. The receptor has been inferred from studies on living membranes. Recently however, Chagas and his associates (*79*) have published the first results of experiments made in an attempt to isolate the receptor. The complete purification has not yet been achieved, but the preliminary results are obviously of paramount importance. Very little is known concerning the storage protein.

We shall examine very briefly some observations on which the theory of Nachmansohn is based.

A. EVIDENCE OF THE EXISTENCE OF THE RECEPTOR

The existence of a receptor has been postulated for many years on the basis of physiological as well as of pharmacodynamic experiments.

Only recently has it been possible to distinguish compounds acting on the receptor from those acting on the esterase.

With the electric organ of *Electrophorus electricus* L., it is possible to measure the esterase activity in the intact tissue using ethylchloroacetate as a substrate (*80*). Acetylcholine cannot be used because of its slow penetration into the membrane. In other words, the discrepancy between the Michaelis constant determined with the homogenized tissue and the Michaelis constant determined on the intact tissue is too large to permit an estimation to be made of the enzymatic activity on the intact tissue.

The use of ethylchloroacetate made it possible to distinguish two types of compounds: (*a*) those affecting the esterase at the time of the block of electrical activity (e.g., eserine* and the tertiary analog of Prostigmine); and (*b*) those without any effect on the esterase activity at the time of the block of electrical activity (e.g., carbamylcholine, decamethonium, and procaine).

These results suggest that the electrical activity may be completely abolished while the esterase is still virtually intact. One must therefore postulate that these drugs have two distinct sites of action, one being with cholinesterase and the other with the receptor postulated in Nachmansohn's cycle. The results do not exclude the possibility that compounds inhibiting the esterase could also affect the receptor. In fact there is experimental evidence to show that, at the time of esterase inhibition, a reaction also occurs with the receptor. This has been shown for eserine (*81*).

B. ACTIVATORS AND INHIBITORS OF THE RECEPTOR

Methylated quaternary ammonium compounds depolarize postsynaptic membranes, while their tertiary analogs do not affect the resting potential (*81, 82*). Depolarization most likely means some change in the structure of the membrane. For this reason, depolarizing compounds (acetylcholine, carbamylcholine, and Prostigmine, etc.) have been called receptor activators (*82a*). They mimic the effects of a physiological stimulus. Those compounds affecting electrical activity without depolarization have been called receptor inhibitors (*82a*) (e.g., curare, eserine, and the tertiary analog of Prostigmine).

The structural modification leading to depolarization may be pictured at the molecular level as a change in the configuration of the receptor molecule. This hypothesis has been given some experimental support by the work of Wilson and Cabib (*83*). Working with two of the proteins postulated in Nachmansohn's cycle, namely with cholinesterase and with choline acetylase, these authors have shown that the entropy of activation is very unfavorable for the tertiary analog of choline both in the hydrolytic and synthetic reactions. They have concluded that the enzymes undergo changes in configuration when reacting with the quaternary compounds, and that such changes do not occur with the tertiary compounds. This is a reasonable explanation since the observed differences cannot be explained on the basis of chemical reactivity or other types of molecular forces (Van der Waals forces, etc.). By analogy, it is then reasonable to assume that the methylated quaternary derivatives depolarize the excitable membrane because they induce a change in the configuration of the receptor.

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* Also known as physostigmine.

C. Effect of Some Ammonium Derivatives on the Cation Fluxes Across an Excitable Membrane

A decrease in the resistance of the membrane means an increase in its permeability. This is well demonstrated by the fact that carbamylcholine, a depolarizing agent, increases the influx of Na across the postsynaptic membranes of the single isolated electroplax of *Electrophorus electricus* L. (*19*).

d-Tubocurarine chloride, on the other hand, decreases the outflux of K. This is consistent with the fact that it is not a depolarizing agent. This observation would therefore suggest that a so-called receptor inhibitor may also change the configuration of the receptor. But, contrary to the effect produced by the activator, the change in configuration leads to a decrease in permeability.

D. Effect of Lipid-Soluble Quaternary Ammonium Ions on Nerve Conduction

An observation generally used against the theory which assigns a role to acetylcholine in nerve conduction is that application of high concentrations of this ester on a nerve is without any effect. This is not surprising when one takes into account the fact that the methylated quaternary ammonium derivatives are insoluble in lipids and may not reach the active site in the conducting membrane (*84, 85*). Recently, quaternary ammonium ions which are lipid-soluble have been developed (*86*). These compounds are derived from physiologically active chloroform-insoluble quaternary ammonium ions by replacing one methyl group by a dodecyl radical. These compounds are more soluble in chloroform than in water and it has been shown that at low concentration they block conduction in the axons of lobsters and crabs and depolarize the isolated electroplax of *Electrophorus electricus* L. In frog's rectus abdominis, they produce a contracture which is reversible and repeatable even when the myoneural junction has been blocked by curare (*87, 88*). In frog sartorius, lipid-soluble quaternary amines produce an irreversible double twitch, which can be compared with the two-peak responses obtained in denervated mammalian muscle and normal and denervated frog muscle by close intra-arterial injection of acetylcholine (*89*). All these observations show that lipid-soluble quaternary amines act on the receptor located in the conducting membrane. This conclusion is reinforced by two additional findings obtained with a myelinated fiber: (*a*) these compounds are antagonized by eserine; and (*b*) they specifically increase the permeability to Na ions (*90, 91*).

V. Energy Requirements

The fact that an action potential is carried along a nerve without any change in amplitude means that energy must be expended somewhere in the process. Electrical activity is indeed accompanied by a breakdown of energy-rich phosphates (*92*) and by increased oxygen consumption (*93*). Nachmansohn (*64*) favors the idea that energy is needed not only to resynthesize the acetylcholine used to induce the change in permeability but also to re-establish the ionic gradients (Fig. 5). The energy released by the hydrolysis of phosphocreatine is indeed more than adequate to account for the total electric energy (*92*). This is not surprising when one considers, as Nachmansohn does, that energy is not only needed for the chemical reactions involved in the cycle of the permeability changes but also in the restoration of the ionic gradients characteristic of the resting conditions. On the other hand, many neurophysiologists consider that the energy needed to induce the permeability change comes from the unequal distribution of ions (mainly Na and K) between the cell and its surroundings. The breakdown of phosphocreatine would then supply the energy to restore the unequal distribution of ions characteristic of the resting state (*43*). We thus have two completely opposite views with regard to the energy required for the actual process of changing the permeability: the supporters of a chemical reaction consider the energy-rich phosphate as the primary source of energy, while the supporters of the theory that the permeability change is a purely physical process believe that the energy needed is derived from the ionic gradients.

VI. Adaptations

A. All-or-None Response

Whatever the exact mechanism of the permeability change, there does not seem to be essential differences throughout the animal kingdom nor at various levels of the nervous system of an animal (*94*). The generation of a bioelectric potential seems to result in the activation of a biochemical system present in any excitable membrane; the ionic concentration gradient then becomes available for the production of an electromotive force. The E.M.F. generated is then used for various purposes, among which transmission and integration of information are the most important.

In the preceding sections we have been dealing essentially with the all-or-none response of some excitable membranes. This approach is necessary because this type of response has been most extensively studied. It should be remembered however that the other type of response, the graded one (*94a*), is the most primitive and fundamental as well as the most generalized type of electrical activity (*95, 96*) (Table IV). The term "primitive" is not associated here with the phylogenetic order of organisms but rather with the first appearance of a mechanism which permits the cell to make use of the concentration gradient to produce an electric current. The all-or-none response appears therefore to be a special development of the general property of excitability which seems to have been evolved as a result of the need for (*a*) central control of peripheral organs and (*b*) transmission between widely separated parts in large organisms. On the other hand, the graded response is adequate for transmission over short distances (neuronic aggregates, for instance) and for integration. A membrane is indeed the site of an integrative process if it does not pass on whatever signal comes to it in a one-to-one relation.

TABLE IV
EXCITABLE MEMBRANES ARRANGED ACCORDING TO THE TYPE OF ELECTRICAL RESPONSE PRODUCED[a]

Type of cell	Type of electrical response
Gland: liver, kidney, salivary, etc.	Slow depolarization accompanies functioning
Protozoan, Plasmodium	Graded
Invertebrate and some vertebrate skeletal muscle	Graded
Electroplax: torpedo, skate (modified end-plate)	Graded
Dendrite, pyramid cell	Graded
Electroplax (electric eel)	Graded, all-or-none
Cell body of neuron	Graded, all-or-none
Nerve fiber	Graded, all-or-none
Vertebrate heart	All-or-none
Skeletal muscle	All-or-none

[a] After Bishop (*95*).

Many electrophysiologists adhere to the idea that the two types of response are fundamentally different. According to their view, the all-or-none response is characterized by a specific increase in permeability to Na followed by a specific increase in permeability to K, while the graded response results from an unspecific increase in permeability to all ions.

This concept has its origin in experiments on the muscle end-plate (*97*), in which an isosmotic solution of K_2SO_4 was used as the bathing solution. Aside from the fact that one has to be careful about generalizing from results of experiments conducted under such unphysiological conditions, it has never been shown that graded responses in muscle or nerve fibers correspond to an unspecific increase in permeability to all ions. Moreover, using Na^{24} and K^{42} as tracers, it has been shown that carbamylcholine increases the influx of Na across the end-plate membrane of the electroplax of the eel, while the same compound seems to be without any effect on the K outflux (*19*).

Another argument favoring the concept of a common biochemical mechanism responsible for the graded response and the all-or-none response is the fact that both are affected in the same way by the same compounds (*86*).

Tentatively one could picture the different types of response obtained as arising from variations in the distribution and number of receptor molecules activated by the stimulus, rather than a matter of radically different types of receptors.

B. Speed of Conduction

Conduction of the action potential along a nerve fiber occurs because the active region is negative with respect to the adjacent part of the fiber. A local electrical circuit is developed which stimulates the neighboring portion of the fiber. The rate-limiting factor in this process is evidently the chemical reaction(s) (triggered by the E.M.F.) leading

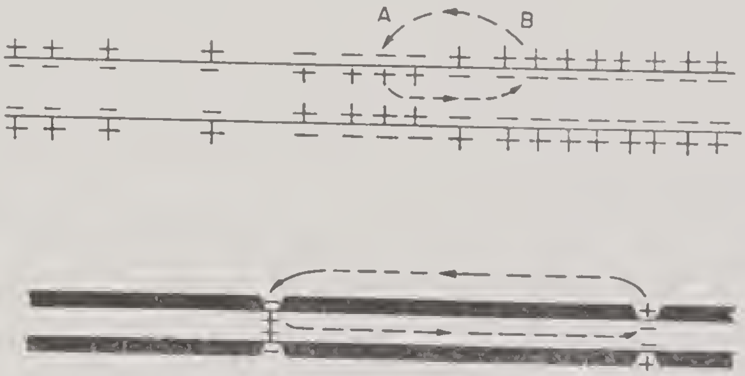

Fig. 6. Schematic representation of the conduction along an unmyelinated nerve fiber (upper sketch) and a myelinated nerve fiber (lower sketch, saltatory conduction). [Hodgkin (*43*.)]

to the change in permeability. Everything being equal, conduction is indeed slower in cold-blooded than in warm-blooded animals.

Speed of conduction is dependent on the *fiber diameter* (*98*). In vertebrates, high speed of conduction (*99*) is achieved through a local interruption of the myelin sheath at the node of Ranvier. During activity, the axonal membrane at each node is activated in such a way that conduction proceeds in a *saltatory mode* (*100, 101*) (Fig. 6).

C. SYNAPTIC JUNCTIONS

Synaptic junctions are any localized regions between one excitable cell and another, across which excitation can be transmitted, e.g., axon to dendrite, nerve fiber to muscle or gland cell, nerve fiber to neurone cell body, sense organ to nerve terminal.

Whether the mode of transmission across such junctions is electrical or chemical is still controversial (*64, 102–104*). However, at present, most neurophysiologists consider transmission to be chemical, i.e., mediated by a specific substance (acetylcholine or adrenaline). Indeed, the highest amount of acetylcholine is found in such cellular regions as the cerebral ganglion of *Octopus* (*105*), the central nerve cord of *Periplaneta* (*106, 107*), the central ganglia of the crayfish (*107, 108*), and the sympathetic ganglia of mammals (*109–111*). Choline acetylase (*112–118*) and cholinesterase (*119–122*) are also highly concentrated at synapses. For the latter enzyme, histochemical investigations confirm these results (*123–125*).

However, the problem of synaptic transmission is not relevant to our discussion since, regardless of mode of transmission, the permeability change is the rate-limiting factor in the generation of the postsynaptic electric current. Moreover postsynaptic potentials, inhibitory or excitatory, are, like the spike, a result of changes in electrochemical potential. For example, inhibitory synapses, when activated, change their permeability characteristics in such a way that the Cl equilibrium potential is attained (*102*).

However, we do not have enough information as yet to decide whether or not these synapses are equipped with receptors chemically related to those present at excitable synapses.

D. ELECTRIC ORGANS IN FISHES

Fishes are the only animals which may occasionally be equipped with a morphological structure adapted to produce an electric discharge. Electric fishes belong to the following genera: *Gymnarchus*, *Mormyrus*, *Electrophorus*, *Gymnotus*, *Malapterurus*, *Astroscopus*, *Tor-*

pedo, Narcine, Raia. The most obvious use of the electric discharge is to stun prey. It has also been shown that some types of discharge could be utilized by the fish for orientation purposes (*126–128a*). The common feature shared by all electric organs is that they are composed of a large number of cells (electroplax), arranged in an orderly fashion so that all of them face in the same direction (Fig. 7). Each electroplax is enclosed within a compartment of connective tissue and embedded in a jelly-like substance, the extracellular ground substance. Nerves are distributed only on one face of the plate, the so-called nervous or innervated membrane (*i* in Fig. 7). This membrane is involved in the generation of an electric current, and recent investigations have shown that there can be little doubt that the mechanism generating the E.M.F. in the various kinds of electroplax is of the same nature as the one operating in nerve and muscle. In *Electrophorus electricus* L. there is indeed direct evidence to prove that the discharge depends on ion movements similar to those observed in nerve and muscle (*33*). A striking histological feature of most types of electroplax is the considerable folding of the nonnervous membrane (*n* in Fig. 7). This membrane has many digitations protruding outwards. They represent an adaptation which improves the efficacy of the discharge by lowering the resistance of the membrane through an increase in surface area (*129*).

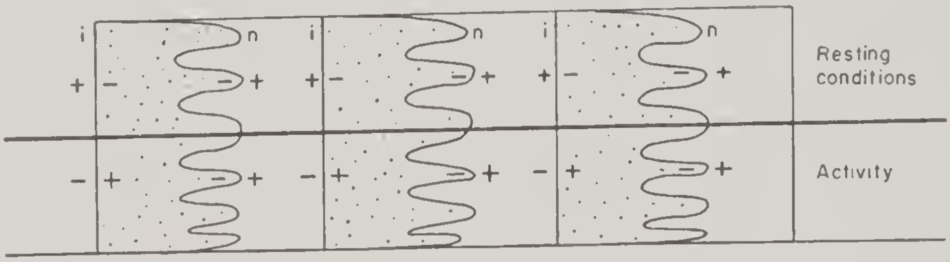

FIG. 7. Diagrammatic representation of the electric organ of *Electrophorus electricus* L. showing how a powerful discharge is produced by the summation of the voltages developed across the nervous membrane (*i*) of each electroplax. *n* is the nonnervous membrane.

The nonnervous face is unable to generate an action potential and its resting potential is only little changed during the spike. In a column of electroplates there is thus a summation of every E.M.F. generated by the nervous face of each electroplax. Figure 7 illustrates how a powerful electric discharge can be produced by the arrangement of many plates in series.

VII. Conclusions

What is generally referred to as the cell membrane is a barrier preventing the intracellular fluid from mixing rapidly with the external medium. This barrier is able to perform secretory work and exhibits various characteristics of specific permeability towards ions. Consequently, an electrical potential difference is built up across the membrane. It is not known at the present time whether the electrical potential difference is of critical survival value or is just an incidental consequence of a more fundamental necessity, i.e., the maintenance of a certain intracellular ionic composition. However it is obvious that excitable structures use the free energy stored as ionic gradients to produce an electric current. This is achieved through the acquisition of a special mechanism enabling the permeability characteristics of the membrane to change for a very short time. Consequently Na enters and K goes out of the cell. Thus the primary event in the production of an action potential is the breakdown of the membrane resistance.

The biochemical system underlying the permeability cycle is not yet fully understood but there is good experimental evidence to show that quaternary ammonium bases are involved in the process.

Transmission of nerve impulses across synaptic junctions, electric discharge in fishes, integration in the central nervous system, conduction along axons, etc. are special aspects of the production of bioelectric currents. Manifestations of activity at these various focuses are not always identical, but the differences are a matter of adaptation, biochemical or morphological, of a primary function, i.e., the use of an ionic gradient as the source of an electromotive force.

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CHAPTER 11

Bioluminescence

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I. Distribution of Luminescence in the Living World

Many textbooks of biology hardly mention the subject of bioluminescence, despite the fact that the ability to produce light is found among representatives of approximately one-half of the twenty-five generally recognized phyla of the animal kingdom, as well as in one (bacteria and fungi) of the nine great divisions of the plant kingdom. In round numbers some forty to fifty different groups can emit light, depending on the classification selected. Details regarding the gross structure, histology, and physiology of their luminous organs will be found in the author's (*1*) book, "Bioluminescence" (1952).

The mere fact that a newly discovered animal is luminous is no guarantee that it is self-luminous. In many cases the individual may have become infected with luminous bacteria and will eventually die as a result of the invasion of its tissues with these parasitic microorganisms. Among other groups it has been established beyond question that symbiotic luminous bacteria are the cause of the luminescence. Frequently the symbiotic bacteria live in a specially developed lumi-

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† Deceased.

TABLE I
LUMINOUS GROUPS WITH REPRESENTATIVE GENERA[a]

Group and Typical Luminous Genera	Habitat	Type of light	Oxygen necessary	Luciferin-luciferase reaction	ATP reaction
Bacteria (*Photobacterium*, *Achromobacter*, *Vibrio*)	MF	I	+	+	−
Fungi basidiomycetes (*Panus*, *Omphalia*, *Pleurotus*)	T	I	+	−	−
Radiolaria (*Thalassicola*, *Sphaerozoum*, *Collozoum*)	M	I	−	−	
Dinoflagellata (*Noctiluca*, *Ceratium*, *Gonyaulax*)	M	I	+	−?	−
Porifera (*Grantia*)	M	I?		−	
Hydroidea					
Hydroids (*Campanularia*, *Obelia*, *Aglaophenia*)	M	I?		−	−
Hydromedusae (*Aequorea*, *Halistaura*)	M	I?	−	−	−
Siphonophora (*Diphyes*, *Hippopodius*, *Agalma*)	M	I?			
Scyphomedusae (*Pelagia*)	M	E?	−	−	−
Pennatulacea (*Cavernularia*, *Pennatula*, *Renilla*)	M	I	+	−	+−
Gorgonacea (*Ceratoisis*, *Primnoisis*)	M	?			
Ctenophora (*Mnemiopsis*, *Beroë*, *Pleurobranchia*)	M	I	−	−	−
Nemertinae (*Emplectonema*)	M	I			
Polychaeta					
Polynoinae (*Acholoë*, *Polynaë*, *Harmothoë*)	M	I	+	−	
Alciopidae (*Calizonella*, *Corybocephalus*)	M				
Tomopteridae (*Tomopteris*)	M	I?		−	
Syllidae (*Odontosyllis*)	M	E		+	
Eunicidae (*Onuphis*)	M	I			
Chaetopteridae (*Chaetopterus*)	M	E	+	−	−
Cirratulidae (*Cirratulus*, *Heterocirrus*)	M	E			
Terebellidae (*Thelepus*, *Polycirrus*)	M	E	+	−	
Oligochaeta (*Microscolex*, *Eisenia*, *Pontodrilus*, *Octochaetus*)	T	E	+	+−	−
Nudibranchia (*Plocamopherus*, *Phyllirhoë*)	M	E?		−	−
Pulmonata					
Ariophantidae (*Dyakia*)	T	I			
Latiidae (*Latia*)	F	E		+	
Prosobranchia (*Planaxis*)	M	I		−	−
Bivalvia (*Pholas*, *Rocellaria*)	M	E	+	+	−
Vampyromorpha (*Vampyroteuthis*)	M	I			
Decapod squid					
Oegopsida (*Watasenia*, *Lycoteythis*)	M	I	+	−	−
Myopsida (*Heteroteuthis*, *Stoloteuthis*)	M	E	+	−	−
Ostracoda (*Cypridina*, *Pyrocypris*, *Conchoecia*)	M	E	+	+	−
Copepoda (*Pleuromma*, *Leuckartia*, *Heterochaeta*)	M	E	+	−	
Amphipoda					
Hyperiidea (*Scypholanceola*, *Streetsia*)	M	I?			

TABLE I (*Continued*)

Mysidacea (*Gnathophausia, Mysis, Siriella*)	M	E			
Schizopoda (*Nyctiphanes, Euphausia*)	M	I		–	
Decapod shrimp					
(*Sergestes, Hoplophorus*)	M	I		–	–
(*Systellaspis, Plesiopenaeus, Heterocarpus*)	M	E		+	–
Diplopoda (*Luminodesmus, Spirobolellus*)	T	I	+	–	–
Chilopoda (*Stigmatogaster, Orya, Geophilus*)	T	E	+	–	
Collembola (*Neanura, Onychiurus*)	T	I			
Hemiptera (*Fulgora*)	T	?			
Diptera (*Ceroplatus, Platyura, Arachnocampa*)	T	I		–	–
Coleoptera					
Lampyridae (*Photinus, Photuris, Luciola*)	TF	I	+	+	+–
Phengodidae (*Phengodes*)	T	I	+		–
Rhagophthalmidae (*Dioptoma*)	T	I			
Drilidae (*Diplocladon*)	T	I			
Drilidae (*Phrixothrix*)	T	I	+	–	–
Elateridae (*Pyrophorus*)	T	I	+	+	+
Ophiuroidea (*Amphiura, Ophioscolex, Ophiopsila*)	M	I	+	–	
Enteropneusta (*Ptychodera, Balanoglossus*)	M	E	+	–	–
Tunicata (*Pyrosoma, Appendicularia, Salpa*)	M	I		–	–
Elasmobranchii (*Etmopterus, Laemargus*)	M	I			
Teleostomi[b]					
Batrachoididae (*Porichthys*)	M	I			
Stomiatoidae (*Echiostoma, Gonostoma, Maurolicus, Polyipnus, Argyropelecus, Chauliodus*)	M	I		–	–
Myctophoidea (*Myctophum, Diaphus, Lampadena, Neoscopelus*)	M	I		–	–

[a] Modified from Harvey (*3*).

[b] See footnote in Section V, p. 578.

nous organ which contains devices for screening their light, or a crude but transparent lens-like mass of tissue for directing the light. Such luminous organs containing luminous bacteria are always present in every individual. They are common in various species of fish.

In other animals in which the light is due to luminous bacteria, for example the myopsid squid, it is difficult to decide whether the bacteria are truly symbiotic or merely parasitic, living in a definite glandular region of the squid without causing a general infection. Some individuals of one species of such squid, *Sepiola intermedia*, may emit no light, although they possess the gland, while other individuals are very distinctly luminous from bacteria living in the gland (*2*).

A survey of the animal kingdom indicates that bacterial infection of an animal is known to occur among certain isopod (*Megaligia, Porcellio*), amphipod (*Talitrus, Orchestia*) and decapod (*Xiphocaridina*) crustacea, among mole-crickets, May flies, midges, ants, and butterfly larvae, probably among myopsid squid. In some cases the infection is lethal, in others not. Symbiosis with luminous bacteria is possible among myopsid squid and quite certain among some eight families of teleost fish, the Macrouridae, Gadidae, Monocentridae, Anomalopidae, Acropomatidae, Leiognathidae, Serranidae, and probably the Saccopharyngidae and Pediculati. Although animals whose light comes from symbiotic luminous bacteria have great biological and a special evolutionary interest, they add nothing to our knowledge of the comparative biochemistry of luminescence.

The self-luminous groups, based on the best present knowledge, are listed in Table I, together with examples of representative genera which have been studied to a considerable extent. Also recorded are: the habitat, whether marine (M), fresh water (F), or terrestrial (T); the type of light, whether intracellular (I) or extracellular (E); the necessity of oxygen for luminescence; whether the luciferin-luciferase reaction can be readily demonstrated; and whether adenosine triphosphate (ATP) is necessary for the luminescent system. In some cases, where the method of light emission is quite different, the groups recorded are as closely related as families.

In addition to the characteristics included in the table, some groups are sensitive to light, which inhibits the luminescence (ctenophores), while others show a day-night rhythm of luminescence and will not luminesce in daytime even if placed in the dark (the dinoflagellate, *Gonyaulax*). Many species exhibit marked fluorescence of the luminous region in ultraviolet light, either before or after the bioluminescence has been excited (*3*).

II. Essential Chemistry of Light Production

Bioluminescence is a chemiluminescence (*4, 5*) in which the energy of a chemical reaction goes to excite some molecule, whose electron is raised to a higher energy level. A quantum of light is emitted on return of the electron to its original ground level (*6*). This situation gives rise to two fundamental questions concerning a bioluminescence: (1) which is the excited molecule; (2) what chemical reaction supplies the energy for emission of light of the particular wavelengths observed in the emission spectrum of the bioluminescence. The light of luminous organisms ranges from red in the railroad worm *Phrixothrix*, to blue-green in bacteria and the ostracod crustacean, *Cypridina* (see Fig. 1). The energy of the quantum, $h\nu$, is 2.6×10^{-12} erg in the deep red and 4.9×10^{-12} erg in the violet. If these values are multiplied by the number of molecules in a gram molecule of material (Avogadro's number, 6.06×10^{23}) we obtain the free energy necessary for a mole of material undergoing chemical change to emit deep red or violet light (the range of the visible spectrum), with intermediate figures for yellow or green light. In terms of calories (1 cal. $= 4.18 \times 10^7$ ergs), the free energy for red emission is about 41,000 cal.; for yellow, 50,000 cal.; for green, 55,000 cal.; for blue, 62,000 cal. (7). It is obvious that for ultraviolet emission considerable energy is necessary. Ultraviolet bioluminescence maxima have not been detected as yet, although they have not been systematically looked for. There might be some infrared emission in the red light of the railroad worm, but again, the spectrum of this species has not been studied.

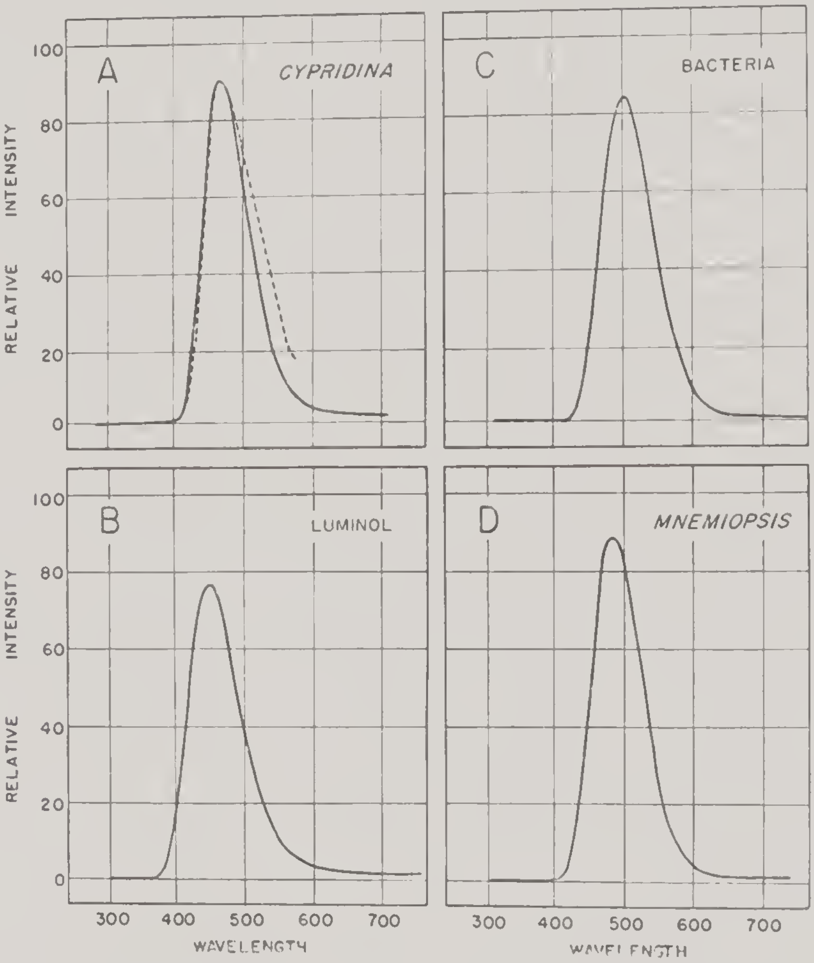

FIG. 1. Spectral energy in arbitrary units plotted against wavelength in millimicrons of the four luminescences indicated. The dotted curve is corrected for spectral sensitivity of the photomultiplier tube which recorded the spectrum. Correction of the other curves would be similar. Luminol is aminophthalichydrazide. *Mnemiopsis* is a ctenophore (from Harvey *et al.*, *53b*).

Early students of bioluminescence realized that some organic compound must emit the light, ever since Radzizewski's (*8*) demonstration that lophine and essential oils (*9*) could be made chemiluminescent in a test tube. Radziszewski (*10*) even applied the find to the medusa, *Pelagia*, holding that an oil was responsible for its light. The words noctilucin or photogen were sometimes applied to the light-emitting substance, but a name which has stuck was used by Dubois (*11*) in 1887 for a heat-stable, dialyzable material, *luciférine.* Luciférine oxidized with light production in the presence of a heat labile enzyme, *luciférase.* These substances were found in the luminous secretion of the mollusk, *Pholas dactylus.* Dubois demonstrated luciferin and luciferase by mixing a cold-water extract of the luminous tissue, allowed to stand until all light disappeared, with a hot-water extract of luminous tissue allowed to cool. The cold-water extract contains luciferase, since all the luciferin has oxidized, and the hot-water extract contains luciferin since the luciferase has been destroyed by heat. This "luciferin-luciferase reaction" has been tested in a number of luminous forms, with the results indicated in Table I. Dubois (*12*) had previously observed the reaction in the elaterid beetle, *Pyrophorus,* but did not use the words luciferin and luciferase at that time. The luciferin-luciferase reaction may be regarded as the fundamental one for light emission.

In recent years accessory substances have been discovered, necessary for light emission in certain forms, the first one being adenosine triphosphate (ATP) in the fire-fly (*13*). This "ATP reaction" is obtained when light results on adding ATP to a cold-water extract of the luminous tissue allowed to stand until all luminescence disappears. It has been tested (*14, 15*) in a number of organisms, also indicated in Table I.

In luminous bacteria, additional accessory substances necessary for luminescence have been discovered, for example a long chain aldehyde, in addition to reduced flavin mononucleotide. The details of the luminescent systems in the best known, organisms, in *Cypridina,* in the fire-fly, and in luminous bacteria, will be considered in subsequent sections, but it is important to note that they are all different and that the name luciferin can now be used only as a generic term, like the word vitamin. Such a generic term is a convenience and the question arises how luciferin should be defined. Concerning this matter the author (*16*) has written: "Rather than placing the emphasis on a limiting factor, or on heat stability or dialyzability or even oxidizability (as an indication of luciferin), as has been done previously, light emission should be the criterion. In the case of luminous organisms requiring dissolved molecular oxygen for luminescence, luciferin may properly be defined as the oxidizable substance supplying molecules capable of absorbing enough excess energy to emit in the visible region. Such a definition implies that some form of luciferin molecule—either free base or acid, either dissociated anion or cation, in reduced or oxidized form, either free or combined with protein, like a prosthetic enzyme group—can pick up the energy of the oxidative reaction in which it is involved. Such a definition does not mean that luciferin is the same substance in different luminous animals, nor does it necessarily designate luciferin molecules themselves as the ones which emit, but it does imply that a related molecule, such as a luciferin-luciferase combination, or an oxidized luciferin molecule, or a molecule of an intermediate step, is the emitter."

The ability to absorb excess energy is particularly characteristic of fluorescent compounds. It has long been recognized that substances which fluoresce by absorbing the energy of radiation are most likely to chemiluminesce by absorbing the energy of a chemical reaction. It is therefore not surprising to find that fluorescence is a characteristic of the light organs of many luminous animals or that the various compounds designated as luciferin in *Cypridina,* in the fire-fly, or in bacteria (see Section IV) are fluorescent in some form. Of particular interest is the ctenophore, *Mnemiopsis,* whose luminous cells show no fluorescence in near ultraviolet light before bioluminescence, but a marked bluish fluorescence after the bioluminescence has ceased (*17*), as if the reaction product was a fluorescent material.

It would be satisfying if one could make the universal statement that dissolved oxygen is always necessary for a bioluminescence. Such a statement would not be true. In 1926 the author (*18*) discovered that certain organisms (the radiolaria, *Thalassicolla* and *Collozoum;* the scyphomedusa, *Pelagia;* the ctenophore, *Beroë*) would luminesce in a medium devoid of oxygen and containing hydrogen and platinized asbestos. Since then the observation has been confirmed for other medusae (the hydromedusa, *Aequorea*) and particularly (*19*) for the ctenophore, *Mnemiopsis,* which can luminesce in a concentration of sodium hydrosulfite which has absorbed all dissolved oxygen and is sufficiently concentrated to keep the dye safranine in the colorless reduced condition. One can only say that these organisms may have previously bound oxygen in a complex from which it cannot be readily removed, but can presumably be activated by luciferase to take part in a luminescent reaction. Table I records the results of a study of the organisms which do or do not require dissolved oxygen for luminescence. It will be noted that the great majority of those tested do require dissolved oxygen. The relation between oxygen concentration and luminescence intensity has been carefully studied by J. W. Hastings in a number of forms, in bacteria and fungi (*20*), in *Cypridina* (*21*), in the fire-fly (*22*), and in *Luminodesmus* (*23*).

III. Fundamental Differences in Plants and Animals

Despite some reports of luminous marine algae and of luminous higher plants, the only certain luminous members of the plant kingdom are the bacteria and the fungi. The light of marine algae is usually due to hydroid colonies established on the alga; the light of the leaves (*24*)* of living angiosperms is usually due to the fungus, *Omphalia flavida*, growing on the leaf; the light of the milky sap of a *Euphorbia*, reported in 1828, was (in the author's opinion) a reflection.

Among fungi, the mycelium of *Xylaria*, of the Ascomycetes, has been described as luminous, but little is known of the conditions under which light appears. Several of the Basidiomycetes are luminous, either mycelium or fruiting body, or both. Thus far the higher fungi have resisted all attempts to obtain luminous cell-free extracts.

Among luminous bacteria, the shape of the cell may be that of a rod, a vibrio, or a coccus, with occasional filaments or "large bodies" appearing in some cultures (*25*). These large bodies increase in number in presence of penicillin (*26*). Various species of luminous bacteria may be gram-positive or gram-negative and may or may not liquify gelatin or ferment various sugars. They may prefer high or low temperature, little salt or 3% salt, i.e., the concentration in sea water. They exhibit definite immune reactions, are attacked by bacteriophage, and undoubtedly exist in many different strains. The important nonluminous mutants will be discussed in the section dealing with evolution of luminescence. All luminous bacteria require dissolved oxygen, both for luminescence and for growth.

The most important difference between the fungi and bacteria on the one hand and animal luminescence on the other is the fact that animals, with few exceptions, luminesce only on stimulation, whereas bacterial and fungal light is continuous and steady, changing only with temperature or food supply or other drastic conditions of the environment. They cannot be stimulated to luminesce. This fundamental difference is the reason for not considering (in this paper) some of the luminescent green dinoflagellates as belonging to the plant kingdom. *Gonyaulax*, for example, behaves like a plant in that it cannot live without light and photosynthesis. However, *Gonyaulax* only luminesces on stimulation and thus exhibits a fundamental animal characteristic.

There are a few animals whose light is not due to luminous bacteria, which nevertheless display a steady luminescence of their photogenic cells quite independent of stimulation. In this category must be placed the eggs and pupae of fire-flies and the adult females of *Phengodes*. In all stages of development, light comes either from wandering cells or from oenocytes (in *Phengodes*) which lack the nerve connections present in larval or adult fire-flies. Dipterous larvae (*1*) have some control over their luminescence but it is nearly continuous, and the diplopod, *Luminodesmus* (*27, 28*), is in the same category, as well as some of the collembola, for example *Onychiurus armatus*, studied by Heidt (*29*). These forms do not respond to electrical stimulation. No luminous bacteria have been demonstrated in any of them, but the fine structure of the luminous cells in all species remains to be determined.

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* The invisible luminescence connected with photosynthesis, studied by Strehler and Arnold (*24*), is not considered a bioluminescence for this review.

The important question whether the chemistry of luminescence differs in plants and animals cannot be answered with certainty at the present time. The luminous system in bacteria is very thoroughly known. As indicated in a subsequent section, the essential constituents are dihydroflavin mononucleotide ($FMNH_2$), a long chain aldehyde, oxygen and luciferase, together with reduced diphosphopyridine nucleotide (DPNH) and DPN oxidase to reduce the FMN which forms during luminescence. This is very different from the system in the fire-fly and the system in *Cypridina*, but it is possible that earthworms may possess a luminescent system similar to that of bacteria. The components have not been obtained in purified condition but it is known that the luminous cells of some earthworms (*Eisenia submontana*) contain large quantities of riboflavin (*30*), not flavin mononucleotide or flavin adenine dinucleotide (*31*). A group of Czech workers believe the light comes from an oxidative reaction involving oxidation of a flavin adsorbed in an oriented layer on the surface of granules. The chemistry involved is discussed in a later section. Further work will be necessary to clarify the situation but at least one animal appears to luminesce somewhat as do the bacteria, by virtue of a compound containing the isoalloxazine nucleus.

One might expect that methods of extracting luminous material from bacteria would work with fungi but such is not the case. Both Hastings (*32*) and Cormier (*32*) have attempted this without success, so that it is not even possible to state at the present time that the fundamental chemistry for luminescence of fungi is the same as that for bacteria. It would be of great interest to know what factor is lacking in the nonluminous strain of *Panus stipticus*, whose genetic behavior when crossed with the luminous strain of *Panus stipticus* has been studied by Macrae (*33*). At the present time, therefore, the chief difference between plant and animal behavior in luminescence has to do with the excitation mechanism. Little is known of this except in the case of the fire-fly, as indicated below.

IV. Well-Known Luminescent Systems

A. The Ostracod Crustacean, *Cypridina*

1. *Luminescent System*

The starting material for biochemical studies on the ostracod crustacean, *Cypridina hilgendorfii,* is the rapidly dried animals, collected in Japan. These organisms are about 3–4 mm. long and possess a gland near the mouth from which the luminous substances are squeezed by muscles into the sea water whenever the animal is disturbed. The dried material will keep indefinitely in a dry atmosphere over $CaCl_2$. Thus far only two substances, luciferin and luciferase, have been found to be necessary for light emission in water containing dissolved oxygen. No salt is necessary, although the amount of light emitted during a luminescent reaction is dependent on the salt content of the medium (*34*) and is in fact quite sensitive to certain ions. The luminescence changes, depending on whether sodium or potassium phosphate buffers have been used at the same pH (*35*).

A large amount of research has been directed to a study of the kinetics of the luminescent reaction and the effects of various substances and factors such as temperature (*36*) and pressure (*37*). These studies have been reviewed by Harvey (*1*) and only research bearing on the chemical properties of luciferin and luciferase can be considered here. Luciferin is determined by the total amount of light emitted under standard conditions and luciferase by the velocity constant of light production from luciferin undergoing oxidation (under standard conditions) as a first order reaction. The Michaelis-Menten constant for the luciferin-luciferase system has been determined by Chase (*38*), and its variation with temperature by Kauzmann and associates (*39*).

2. *Luciferin*

Historically* *Cypridina* luciferin has been called a peptone (Harvey, 1919, *40a*), a phospholipid (Kanda, 1930, *40b*), a polyhydroxybenzene derivative (Anderson, 1936, *40c;* Korr, 1936, *40d*), a hydroquinone-like compound with a ketohydroxy side chain (Chakravorty and Ballentine, 1941, *40e*), a flavoprotein and pyridine nucleotide (Johnson and Eyring, 1944, *40f*), and a chromopolypeptide (Mason, 1952, *41*). The hydroquinone type of structure was championed by the Dutch group of investigators (Kluyver *et al.*, 1942, *41a*; van der Kerk, 1942, *41b*), particularly for bacterial luciferin, in the form of 1,4-dihydroxynaphthyl-2-hydroxymethylketone. It seems quite certain that this compound cannot be *Cypridina* luciferin, since no light appears when it is mixed with luciferase (Johnson *et al.*, 1949, *41c*; Spruit, 1949, *41d*), although related naphthohydroquinones have not been ruled out.

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* References to historical papers are from the bibliography of E. N. Harvey's "Bioluminescence" (*40*).

Space does not permit a review of the complete history or the evidence for and against the above hypotheses. The important findings concerning the chemistry of *Cypridina* luciferin, facts which appear to have been established without question, may be summarized as follows:† Analyses of luciferin, prepared by Anderson's (*43*) benzoylation procedure with two cycles of benzoylation, indicate the presence of nitrogen (*44*), confirmed by the carbylamine test (*44a*) and by a ninhydrin-positive reaction after (but not before) acid hydrolysis of material purified both by paper chromatography (*41, 45*) and by paper electrophoresis (*45*). Recent studies indicate no phosphorus in doubly cycled luciferin by the molybdenum blue reaction of Fiske and Subbarow as modified by Sumner. The reaction is capable of detecting one part in 5 million of phosphorus. No carbohydrate has been found by the anthrone test, capable of detecting one part in 900,000 of starch. Control tests with minimal amounts of phosphate and glucose indicate that the two reactions were carried out properly. Luciferin thus appears to contain C, H, O, and N as the primary constituents. No sulfur has thus far been detected.

Anderson's benzoylation method, yielding a compound nonluminescent with luciferase and nonoxidizable with oxygen, but hydrolyzed to active luciferin by HCl, indicates an SH, OH, NH, or NH_2 group. Acetic anhydride will form a similar nonactive acetyl-luciferin (*43*).

The doubly cycled luciferin has been found to combine irreversibly with cyanide, possibly indicating cyanhydrin formation with aldehyde or ketone groups (*46*). With azide, luciferin forms a reversible combination (*47*). A. M. Chase (unpublished) found no reaction with 2,4-dinitrophenylhydrazine.

Additional chemical tests have been made by Mason and Davis (*48*) which led them to the conclusion that a ketophenol and a quinonoid structure are ruled out, but the amino group appears to be present and it is possible that the oxidation-reduction change in luciferin involves the NH group.

Anderson (*49*) and Korr (*50*) by indirect methods have placed the redox potential of luciferin at about +0.26 volt at pH 7. The rapid oxidation by ferricyanide has been made use of by Chase (*51*) in estimating that the combining weight of luciferin is between 250 and 570,

† An early summary of luciferin properties will be found in A. M. Chase (*42*).

while a similar calculation from combination with cyanide indicated 800 to 2400 (*46*). Such calculations are subject to revision, depending on the amount of impurity present.

Purified luciferin undergoes rather rapid spontaneous oxidation in ultraviolet light shorter than 300 mμ and in near ultraviolet and blue light if photosensitizers such as fluoresceine, eosine, and riboflavin are present.

Recent methods of purification and approach to the structure of *Cypridina* luciferin, such as infrared spectroscopy, chromatography, and acid hydrolysis have been initiated by Mason (*41*) and Mason and Davis (*48*). Infrared spectroscopy, carried out with thin films of dried, doubly cycled luciferin, has revealed strong absorption bands at 3250, 2825, 1680, 1625, and 1510 cm.$^{-1}$, collectively indicating the amide bond as it occurs in peptides or in cyclic ureides. Acid hydrolysis of paper-chromatographed luciferin has yielded a yellow pigment and some eight amino acids, a result which led Mason (*41*) to the designation of *Cypridina* luciferin as a chromopolypeptide of a cyclic nature. A polypeptide structure of luciferin would place it in the group with a number of naturally occurring biological compounds, particularly the antibiotics, gramicidin and polymyxin, but with the addition of a color group. The solubilities of *Cypridina* luciferin are surprisingly similar to the cyclopeptide gramicidin, with the one exception that luciferin is more soluble in water. The ultraviolet absorption spectrum of gramicidin is, however, dissimilar to that of *Cypridina* luciferin (Chase, unpublished measurements).

The more recent paper and cellulose column chromatography research of Tsuji, Chase, and Harvey (*45*) has indicated that impurities still remaining after the Anderson benzoyl method of preparation can be to a considerable extent removed by chromatographic separation, and the resulting product, when hydrolyzed and analyzed by the Moore and Stein method, yields in order of abundance: phenylalanine, arginine, isoleucine, glycine, leucine, valine, and traces of aspartic acid, threonine, serine, glutamic acid, and methionine. There was also considerable ammonia.

The extensive ultraviolet spectrophotometric study of material prepared by a new method of Tsuji (*52*) has indicated that *Cypridina* luciferin exhibits spectral changes in acid and alkali and in reduced and oxidized condition (Fig. 2). It was long ago established by Chase (*53*) that luciferin absorbs in the blue region of the visible spectrum at 435 mμ and hence has a yellow color. After a preliminary shift to an absorption peak at 465 mμ at pH 7, the solution becomes colorless on a complete oxidation. Although *Cypridina* luciferin undergoes nonluminous spontaneous oxidation in oxygen-containing water, or on addition of mild oxidizing agents like ferricyanide, no difference in the spectral absorption changes which occur can be detected during the spontaneous

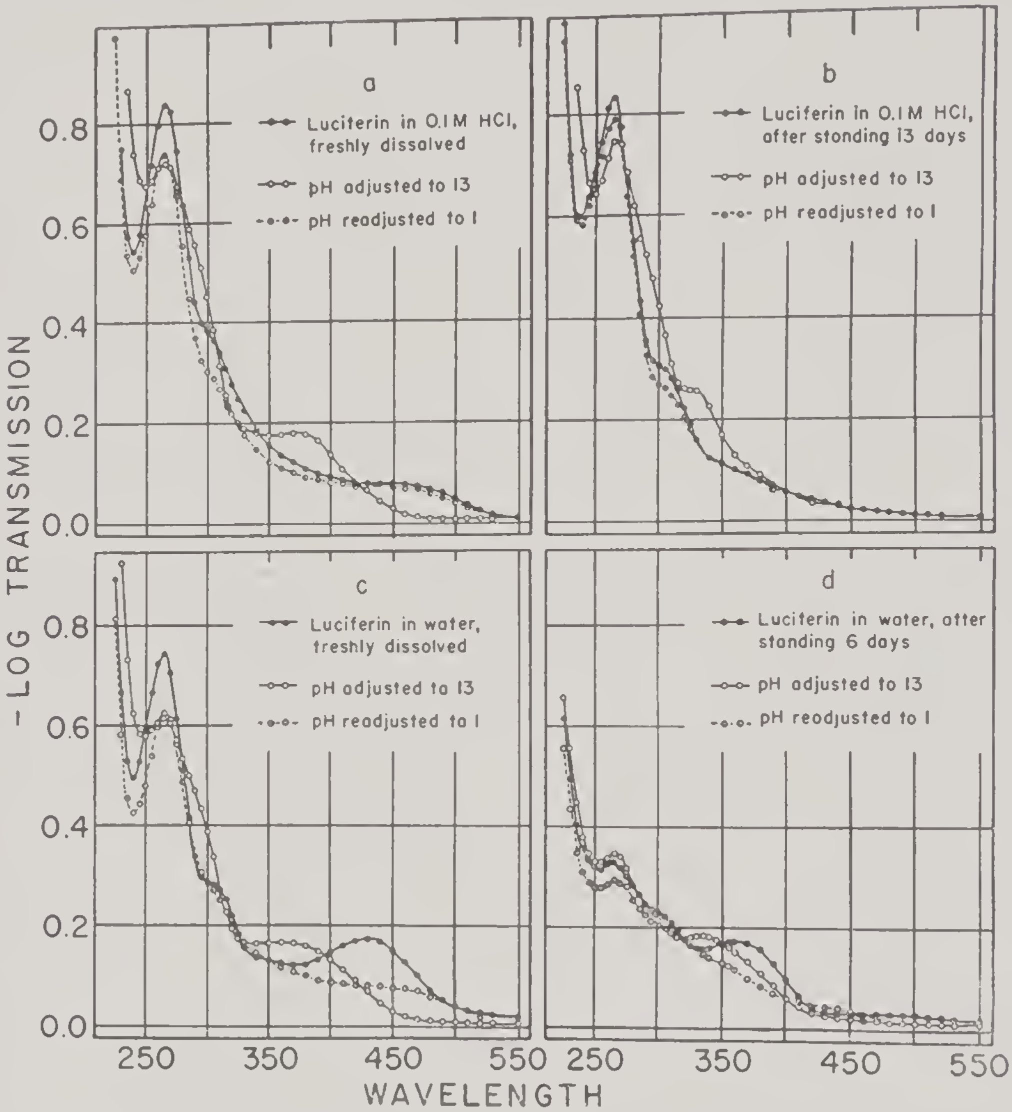

FIG. 2. Ultraviolet absorption of purified *Cypridina* luciferin showing changes in acid and alkali (after Tsuji, 52). Wavelength in millimicrons.

oxidation as compared with the luciferase oxidation or the ferricyanide oxidation (52). The latter two are merely more rapid. These changes are illustrated in Fig. 3.

When luciferin oxidizes spontaneously without luminescence it can be partially reduced by various procedures which add hydrogen to the molecule (*53a*). On standing or after more rapid oxidation in presence of luciferase, reduction of the oxidized luciferin cannot be effected by simple addition of hydrogen. It is possible that the preliminary absorption shift from 435 mμ to 465 mμ represents a reversible redox change with a redox potential of +0.26 volt, near hydroquinone-quinone, whereas later an irreversible oxidative step occurs leading to further changes in the absorption spectrum. The preliminary 435 to 465 mμ shift does not occur in acid solutions (*N*/10 HC1).

Luciferin is yellow in color in the living *Cypridina* and rather weakly yellow fluorescent as compared with such markedly fluorescent substances as flavins. The absorption band for excitation of fluorescence has not been identified with certainty, but the weak fluorescence may be correlated with low absorption in the near ultraviolet region, an absorption which becomes much less as oxidation of luciferin proceeds. Connected with this lower absorption the fluorescence of oxidized luciferin practically disappears (*45*). Another factor in the slight fluorescence of luciferin is the fact that the yellow color nucleus (presumably the fluorophore group) is part of a polypeptide. Flavins largely lose their fluorescence when combined with protein. The emission spectrum of

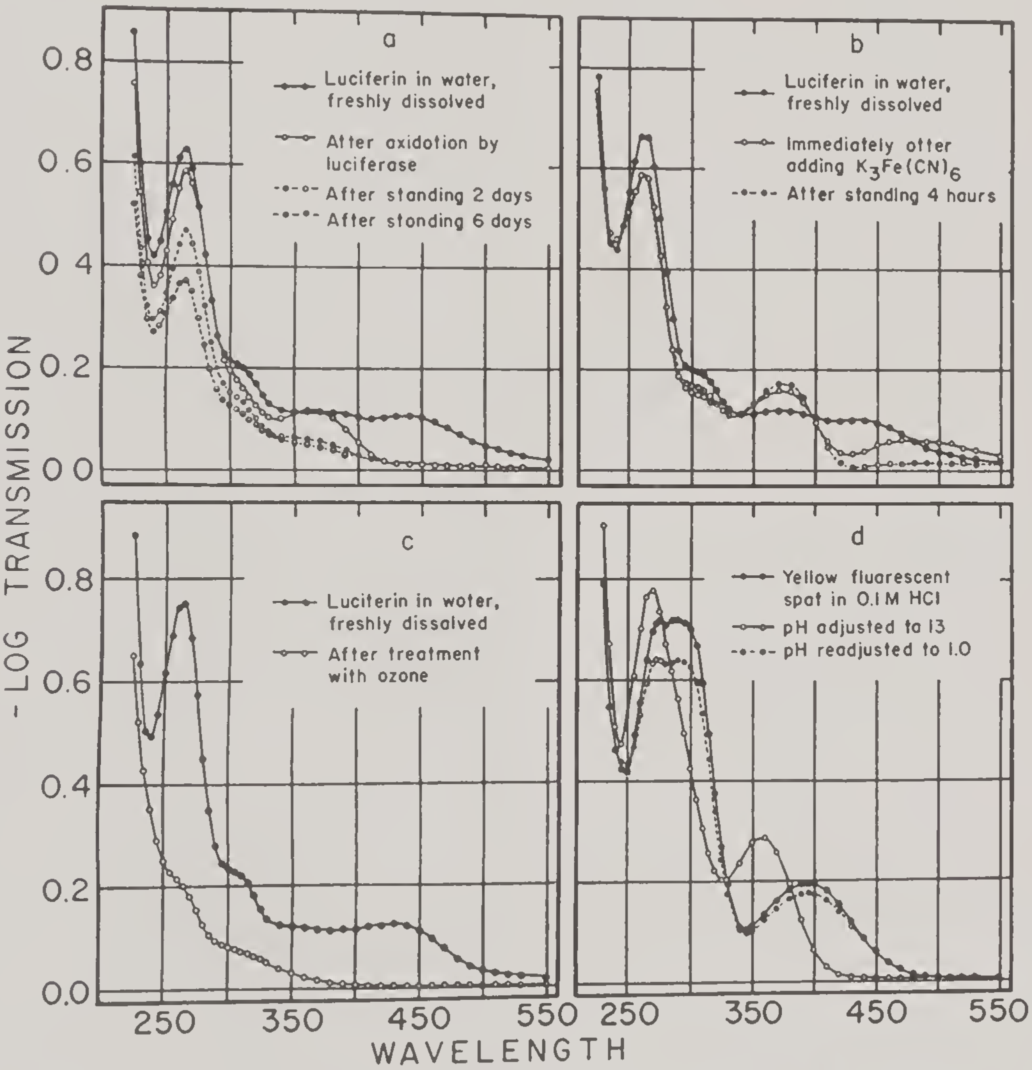

FIG. 3. Ultraviolet absorption changes of purified *Cypridina* luciferin during (a) spontaneous and luciferase oxidation; (b) after ferricyanide oxidation; (c) after treatment with ozone; (d) a nonluminous yellow pigment from *Cypridina* in acid and alkali (after Tsuji, *52*). Wavelength in millimicrons.

Cypridina luminescence has a maximum in the blue, as indicated in Fig. 1.

In view of the ready spontaneous oxidation of luciferin without luminescence, chemical study and purification of the material should be carried out in absence of oxygen or at low temperature. To offset the difficulties of oxidation, the work of Marfey, Craig, and Harvey (*54*) was carried out with benzoyl-luciferin, which does not oxidize readily, and can be hydrolyzed to luciferin before testing. The partially purified benzoylated luciferin prepared by Anderson's method (*43*) from dried-benzene-extracted *Cypridina* has been subjected to counter-current distribution, using a water-methanol-chloroform-cyclohexane solvent system and the various fractions (after hydrolysis) tested for luminescence with luciferase, for fluorescence, for optical density at 272.5 mμ (the region of greatest absorption), for total solid and for amino-acids (after hydrolysis) by paper chromatography. It was found that several regions of the distributed material would luminesce with luciferase but one region was particularly bright. On further hydrolysis of this purified luciferin by strong acid, a yellow pigment (perhaps two-thirds of the material) and 10 or 11 amino acids (arginine, leucine, glutamic acid, alanine, valine, isoleucine, phenylalanine, tyrosine, aspartic acid, serine or glycine, and possibly cystine) were found. Thus the counter-current distribution method of purification has also indicated that luciferin is a chromopolypeptide. Some of the other fractions have yielded similar amino acids (in different proportions) and might represent oxidized luciferin or slightly different composition of the polypeptide combined with the color nucleus. Important future work will indicate whether the yellow chromophore alone will luminesce after the polypeptide component has been removed by acid hydrolysis or whether it must be combined with the latter in order to be luminescent.

Filter paper electrophoresis (*45*) has indicated that luciferin is positively charged, acting like a cation, at pH values 2.5, 4, and 5.8 but more alkaline values have not been tested because of the rapid oxidation of the material. Therefore an isoelectric point has not been determined.

When *Cypridina* luciferin oxidizes in the air or in presence of mild oxidizing agents like ferricyanide, no light has thus far been detected. The only conditions under which a luminescence has appeared without luciferase is on mixture of luciferin in acid solution with strong oxidizing agents such as H_2O_2, inorganic and organic peroxides, permanganates, and bichromates (*55, 56*). However the intensity of this luminescence is very much weaker than that with luciferase. It has also been observed that methanol solutions of luciferin exposed to far ultraviolet light will luminesce for some time after exposure, an apparent phosphorescence, which has been traced to the formation of peroxide in the solvent by the ultraviolet light (*57, 58*).

In general chemical behavior, *Cypridina* luciferin behaves like a weak base which forms a salt with acids (*54*). The manipulations described above have mostly involved the salt. The free base is readily autoxidizable, with loss of the ability to luminesce in presence of luciferase. Unlike the free acid of fire-fly luciferin, both salt and free base of *Cypridina* luciferin are readily soluble in water (neutral, acid, or alkaline) as well as in the lower aliphatic, monohydric alcohols from methyl to butyl. The free base (and probably the salt) are soluble in glacial acetic acid and dimethylformamide. The salt is insoluble in petroleum ether, carbon tetrachloride, benzene, ether, and chloroform, while the free base is slightly soluble in the first four solvents, more soluble in chloroform, ethyl acetate, and acetone.

Benzoyl luciferin, on the other hand, is very soluble in chloroform, ethyl acetate, acetone, and the lower alcohols, as well as in acetonitrile, dioxane, glacial acetic acid, and dimethylformamide. It is soluble in ether, somewhat soluble in benzene, less so in carbon tetrachloride and very slightly soluble in petroleum ether; insoluble in water, either neutral, acid or alkaline.

In summary, the results (*54*) of two counter-current distributions of benzoyl luciferin, using quite different combinations of solvent in the two cases, have indicated that luciferin (determined by hydrolysis of benzoyl luciferin and luminescence with luciferase) is distributed in a wide range of fractions from beginning to end of a 1000-tube series, but that the most concentrated benzoyl luciferin is to be found in the methanol-water-rich phase as contrasted with a cyclohexane-ethyl acetate-rich phase or a cyclohexane-chloroform-rich phase. When the amino acid content of the seven fractions is tested, the 10 or 11 amino acids previously mentioned are found in all fractions but the amount of each amino acid appears to vary in the different fractions. Tsuji (private communication) has observed that "chromatographically pure" luciferin, prepared in different ways, has a somewhat different amino acid content (as determined by the Stein-Moore method), and also slightly different absorption spectra. The behavior of luciferin is such as to indicate that a yellow color group is combined with a number of amino acids, forming a cyclic chromopolypeptide (*41*), but that the amino groups may vary, giving rise to a number of different *Cypridina* luciferins all of which can chemiluminesce in presence of luciferase. It is possible that the function of a polypeptide structure is to facilitate combination with luciferase.

The *Cypridina* luciferin behavior can be compared with that of the actinomycins, chromopolypeptide antibiotics, containing a phenoxazinone nucleus, brick-red in the oxidized and pale-yellow in the reduced condition, combined with a side chain of two cyclic polypeptides involving lactone linkages between hydroxyl group of threonine and terminal carboxyl group of *N*-methyl valine. The make up of this side chain has been found to vary, giving rise to a number of different actinomycins, all of which have antibiotic qualities but to different degrees (*58a*).

In very recent work, Shimomura *et al.* (*58b*) have reported crystallization of *Cypridina* luciferin as orange-red needles, melting at 182–195° (darkening at 175°), easily soluble in methanol and ethanol, soluble with difficulty in water and acetone, and insoluble in ether and benzene. The ninhydrin test was negative. Phosphorus and sulfur could not be detected, and the elementary composition given (private communication) as $C_{21}H_{26\text{-}28}O_2N_6 \cdot 2HCl$.

When an aqueous solution of luciferin crystals stands in the air the color changes from yellow to an intermediated red compound and then colorless. Chromatography reveals two oxidation products, oxyluciferin A and B. Spectral absorption curves have been obtained similar to those of Chase and of Tsuji. The intermediate red compound, readily obtained with potassium ferricyanide, would not luminesce with luciferase. Oxyluciferin A appeared as pale yellow needles but oxyluciferin B did not crystallize. In the presence of luciferase and with light production, a small amount of oxyluciferin B was formed, but the product was mostly oxyluciferin A.

Luciferin could be hydrogenated with Adams platinum oxide catalyst, resulting in a colorless hydroluciferin with an ultraviolet absorption spectrum similar to tryptamine.

Acid hydrolysis of luciferin afforded glycine, isoleucine, and a fluorescent substance. Hydrolysis with $Ba(OH)_2$ produced glycine, isoleucine, a strong ninhydrin-positive substance, a fairly large amount of anthranilic acid and a small amount of indole or indoleacetic acid, but no tryptophan.

It is too early to speculate on the structural make up of *Cypridina* luciferin but the results seem to indicate that the yellow aqueous solution of a compound with a relatively low molecular weight (470) and relatively few amino acids will luminesce in the presence of oxygen and *Cypridina* luciferase. Five hundred grams of dry whole *Cypridina* powder yielded 3 mg. of crystals, whose luminescent activity by weight was 37,000 times more intense than the starting material.

3. *Luciferase*

Less is known of the chemical make-up of *Cypridina* luciferase, although its kinetics is well understood from the work of A. M. Chase and others. Purification from the dry *Cypridina* powder extract has been carried out by McElroy and Chase (*59*). A water extract at low temperature is precipitated with HCl at pH 4.5 and the precipitate removed and discarded after centrifuging in the cold. The extract is immediately readjusted to a pH of 7.5 with NaOH, and cold acetone slowly added at 0° to 35% by volume, the mixture kept at —5° for 15 minutes, and the precipitate discarded after centrifuging. Additional cold acetone is then added to make 55% and the active precipitate removed and dissolved in cold water. Repeated fractionation with $(NH_4)_2SO_4$ at pH 7.5 and 40 to 65% saturation gives material with a specific activity (relative rate constant milligrams protein) 100 times that of the crude extract. Further purification by adsorption on calcium phosphate gel and elution with 10% $(NH_4)_2SO_4$ at pH 7.3 will increase the specific activity but greatly lower the per cent recovery.

This purified material gives a bright luminescence with purified luciferin and does not require the presence of Mg or Mn ions. Its spectral absorption is entirely in the ultraviolet, with a peak at about 280 mμ, hence not connected with flavin or purine or nucleic acid components. The material is slightly more resistant to heat inactivation than crude luciferase but the kinetics of inactivation is the same as with the crude material. The activation energy for destruction of the crude material at pH 6.8 between 40° and 55° is about 57,000 cal. Heat inactivation is faster at lower pH (*60*).

Paper electrophoresis of acetone — $(NH_4)_2SO_4$ purified luciferase (*61*) at seven pH values between 2.38 and 6.18 has indicated an apparent isoelectric point at 3.28, an unusually low value for enzymes. In the Spinco analytical ultracentrifuge, Chase (*62*) observed two high molecular components. The one with a lesser sedimentation constant (2.9 to 3.6 Svedberg units) was identified with luciferase. If the molecule is assumed to be spherical and without water of hydration, a minimum value for the molecular weight is in the range of 35,000–40,000. More recently the diffusion constant, *D*, for luciferase has been determined by Fedden and Chase (*63*), using the Northrop-Kunitz porous disk method. When sintered glass was used for the disk, average values of $D = 7.41 \times 10^{-7}$ cm. 2/seconds at 20° were obtained. The radius of the molecule from the Einstein equation, assuming a spherical shape and no water of hydration, was 2.9×10^{-7} cm. and the molecular weight 80,500. By use of the Svedberg equation and the most probable sedimentation constant and diffusion constant values, Fedden and Chase have calculated a molecular weight for *Cypridina* luciferase of between 45,000 and 37,000, depending on whether a partial specific volume of 0.75 or 0.70 is assumed.

The effect of a number of inhibitors, general and specific, on the activity of the *Cypridina* luminescent reaction has been studied. Giese and Chase (*46*) showed that cyanide did not affect luciferase but combined irreversibly with luciferin. Chase interpreted data on effects of azide, as indicating reversible combination with luciferin rather than with luciferase (*47*). Johnson and Chase (*64*) demonstrated a reversible inactivation of luciferase by urethane and by various sulpha drugs and *p*-aminobenzoic acid, whose effects are partially additive with sulfanilamide. An extended study by Chase (*65*) of a series of monohydric alcohols (with from one to six carbon atoms) has indicated reversible inhibition, with each additional carbon increasing effectiveness two to three times. This behavior has been found quite regularly for biological activity of similar series of aliphatic compounds. Experiments with *n*-butanol indicate a competitive effect of such a nature that the luciferase-luciferin complex is more tightly bound than the luciferase-butanol complex (*66*).

Little can be said concerning the chemical nature of luciferase except that it appears to be a simple protein, perhaps an albumin, without prosthetic groups such as porphyrins, flavins, or pyridine nucleotides. At least the absorption bands of these compounds do not appear in the absorption spectrum of purified material. Unlike fire-fly luciferase, *Cypridina* luciferase is not a globulin, as it is soluble in water free of salts. It is digested by trypsin (*68*) and will give rise to antiluciferase in the blood of rabbits, an observation of Harvey and Dietrick (*69*), recently confirmed by Tsuji (*70*).

B. The Fire-fly

1. *Chemical Reactions*

As early as 1916, Harvey (*71*) found that a hot-water extract of the lanterns of lampyrid fire-flies (*Photinus* or *Photuris*) allowed to cool, would result in luminescence if mixed with a cold-water extract of the lanterns whose luminescence had disappeared. This apparently classical luciferin-luciferase reaction has turned out to be due, not to luciferin in the boiled extract, but to adenosine triphosphate (ATP). As shown by McElroy (*72*), in these particular genera of lampyrids, luciferin disappears in boiled extracts but ATP remains and is responsible for the apparent luciferin-luciferase reaction.

Subsequent investigation by McElroy and collaborators has shown that light production in lampyrid fire-flies requires the presence of adenosine triphosphate, luciferin, Mg, luciferase, and oxygen dissolved in water. Depending on the species of fire-fly studied, the ATP, or the luciferin, or the luciferase may disappear first in water extracts (*73*), but when all five substances are present, light will always appear. None of these substances are specific for any one species of lampyrid. The luciferin from one species will react with luciferase from another and vice versa.

The chemistry of the fire-fly luminescent system has been studied intensively during the past ten years by McElroy and his group at Johns Hopkins, and is well summarized in the McElroy and Green paper (*74*) and in the Harvey Society Lectures for 1957 (*75*). The sequence of reactions in fire-fly luminescence may be visualized as follows:

$$LH_2 \text{ (luciferin)} + ATP \rightleftharpoons LH_2{\cdot}AMP \text{ (adenylluciferin)} + POP \text{ (pyrophosphate)}$$

The adenylluciferin is regarded as active luciferin which can emit light by passing through a peroxidation, with subsequent breakdown to adenyloxyluciferin, water, and adenylic acid (AMP):

$$LH_2{\cdot}AMP + O_2 \rightarrow L{\cdot}O{\cdot}O{\cdot}H_2{\cdot}AMP$$

$$L{\cdot}O{\cdot}O{\cdot}H_2{\cdot}AMP + LH_2 \text{ (or AMP)} \rightarrow L{\cdot}AMP^* \text{ (adenyloxyluciferin)} + 2H_2O + L \text{ (or AMP)}$$

The adenyloxyluciferin molecule is probably the excited molecule (marked with an asterisk), which emits its excess energy as a quantum of yellow ($\lambda = 550$ mμ) light.

$$L{\cdot}AMP^* \rightarrow \text{Light} + L{\cdot}AMP$$

It is now possible to synthesize adenylluciferin by chemical procedures, starting with crystalline luciferin, and to show that this compound will give light in presence of luciferase and oxygen in solution. Under anaerobic conditions adenylluciferin is hydrolyzed spontaneously to luciferin and adenylic acid (*74*).

The adenyloxyluciferin can be shown to be a potent inhibitor of luciferase. It breaks down spontaneously as a dark reaction to adenylic acid and oxyluciferin after dissociation from the enzyme, luciferase.

$$L{\cdot}AMP \rightarrow L + AMP$$

The oxyluciferin may be transformed to a blue pigment or may combine with ATP to give L•AMP + POP, both dark reactions. The pyrophosphate may also break down to inorganic phosphate since there is abundant pyrophosphatase in fire-fly lanterns.

The active group in luciferin is a COOH group, which can react with ATP in presence of luciferase in the same way that acetic acid reacts with ATP to give acetyladenylic acid and pyrophosphate.

$$CH_3COOH + ATP = \text{acetyl-AMP} + POP$$

Just as acetyl-AMP will react with coenzyme A to produce acetyl coenzyme A + AMP, so oxyluciferyl coenzyme A and adenylic acid can be formed. Moreover oxyluciferylcysteine or oxyluciferylglutathione can also be formed by treating oxyluciferyl coenzyme A with cysteine or glutathione, analogous to acetylcysteine or acetylglutathione, formed by treating acetyl coenzyme A with cysteine or glutathione. These reactions can be followed in a test tube by fluorescence emission, which is marked in oxyluciferin and oxyluciferylcysteine but very weak in the oxyluciferyl-coenzyme A combination. Such reactions as the above explain the effect of coenzyme A in increasing luminescence in solution, since this substance removes the L·AMP which is inhibiting the enzyme surface.

A recent paper by Airth, Shodes, and McElroy (*75a*) has furnished additional quantitative details concerning the role of coenzyme A in fire-fly luminescence. There is no doubt that adenylluciferin (active luciferin, LH_2·AMP) corresponds to *Cypridina* luciferin. LH_2·AMP alone in solution with fire-fly luciferase and oxygen can produce light, but the emission stops as a result of oxyluciferin formation and inhibition of luciferase. However, the ability of fire-fly luciferase to catalyze the formation of adenyloxyluciferin from oxyluciferin (L) and ATP is not inhibited. Although no light is produced, the reaction can be followed quantitatively by the change from a highly fluorescent substance (L) to a low fluorescent substance (L·AMP). It is the COOH group of luciferin or of oxyluciferin which combines with a phosphorus hydroxyl group of ATP to form the corresponding adenyl compounds by removal of water. This combination can be brought about by a specific dehydrating agent such as dicyclohexylcarbodiimide in absence of luciferase.

The various oxyluciferin-CoA or cysteine or glutathione complexes can also be followed fluorimetrically. In addition each complex has a distinctive R_f value under special conditions of chromatography.

No redox potential of the fire-fly luciferin-oxyluciferin system has as yet been measured. The oxyluciferin cannot be reduced by hydrosulfite or platinum and hydrogen. Possibly a second rapid irreversible oxidative step occurs, since elementary analysis indicates that oxyluciferin contains two less hydrogen atoms than luciferin. Since the removal of two hydrogens corresponds to a free energy change which is probably not greater than 40–50 kcal., it will not account for the emission maximum of fire-fly (*Photinus pyralis*) luminescence in the yellow ($\lambda = \mathrm{m}\mu$), which requires 52,000 calories. For that reason a peroxidation step is assumed between the luciferin → oxyluciferin change. It is probable that the enzyme luciferase is in some manner involved in this change. A peroxidation will liberate approximately 75 kcal. and it is commonly observed that chemiluminescence of a number of organic compounds can be obtained with organic peroxides and with H_2O_2.

The function of the ATP is probably to form an active intermediate with luciferin, adenylluciferin, capable of rapid oxidation with molecular oxygen, which results in formation of an organic peroxide radicle. A peroxidation by the radicle of a second active luciferin molecule would be the essential reaction for the excitation process. The high energy of one phosphate bond may also go to raising the level of excitation of the adenylluciferin molecule. The function of the magnesium appears to be the formation of a chelate structure to bind adenosine triphosphate to fire-fly luciferase where its combination with luciferin can take place, forming adenylluciferin and pyrophosphate.

The luciferase of the fire-fly thus appears to be the same type of enzyme as the acetate-activating enzymes which involve coenzyme A. No heavy metal is contained in fire-fly luciferase and no prosthetic group, unless luciferin is to be considered a prosthetic group. Luciferase of the fire-fly is not a flavoprotein, as is bacterial luciferase, although the flavin content of fire-fly lanterns is high (*76, 77*). In fact the fire-fly lantern appears to contain the various substrates and enzymes of the oxidative respiratory cycle in unusually large amounts, for example pyridine nucleotides. Coenzyme A and pyrophosphatase are also present in large amount.

2. *Luciferase*

Both of the more complex compounds (luciferin and luciferase) of the fire-fly luminescent system have been crystallized, although their structural make up is not completely known. The enzyme luciferase was crystallized first by Green and McElroy (*78*), in 1955, and luciferin in 1956, by Bitler and McElroy (*79*). Details will be found in the original papers; only an outline of the method can be given here.

The essential procedure for luciferase is extraction of the dried, powdered, acetone-washed lanterns (30 gm. or 6000 fire-fly lanterns) with 10^{-3} *M* versene at pH = 7.8 in the cold, and adsorption of the luciferase on calcium phosphate gel, from which it is eluted with 20% $(NH_4)_2SO_4$. The versene chelates heavy metals which may be present. Pyrophosphatase and luciferin remain in the original extract. The eluate from the calcium phosphate gel is then fractionated with $(NH_4)_2SO_4$. The most active precipitate of luciferase appears at 60% saturation (60% of 4.1 *M* $(NH_4)_2SO_4$). Crystallization occurs when the precipitated luciferase is dissolved in weak salt solution (0.15 *M* NaCl and 0.02 phosphate buffer at pH = 7.3) and dialysed against 0.01 *M* NaCl + 0.002 Na_2HPO_4 + 0.001 versene at pH = 7.3.

Fire-fly luciferase is a euglobulin and long crystal needles or rosettes appear as the salt dialyzes away, as shown in Fig. 4. The material is homogeneous on electrophoresis, with an isoelectric point at pH 6.2–6.3, and also homogeneous in the analytical ultracentrifuge. The sedimentation constant is 5.6 at 25° in 0.15 M NaCl. Diffusion constants have not been determined, but the molecular weight must be around 100,000.

The ultraviolet absorption curve shows a peak at 270 mμ and a slight rise at 258 mμ which might be due to combined or adsorbed luciferin. The temperature optimum for luminescence is 23°, with reversible denaturation above this temperature and irreversible denaturation beginning at 35°. The pH optimum is at 7.8. With low ATP concentrations, high concentrations of Mg inhibit and at low Mg concentrations, high ATP inhibits. Increasing amounts of luciferin give a typical Michaelis-Menton substrate-enzyme concentration curve. The function of luciferase is to form adenylluciferin from ATP, and to combine oxygen as an intermediate peroxide, thereby assuring that enough energy will be available to account for the yellow luminescence of these organisms. It is this aspect of luciferase action which makes it a light-producing enzyme.

3. *Luciferin*

Preparation of crystalline fire-fly luciferin (Fig. 5) starts with the same acetone powder (dried, powdered fire-fly lanterns washed with cold acetone which is then removed by evaporation) as used in luciferase preparation. Seventy gram samples (about 15000 fire-fly lanterns) are worked up, first by extracting with hot water to inactivate the luciferase. The pH is then adjusted to 3, the precipitate discarded and the acid solution extracted by four shakings with ethyl acetate (EA), which removes luciferin. The EA is then concentrated and passed through a column of celite. The luciferin can be followed by its fluorescence on the celite. After removing the EA, the luciferin is eluted from the column with alkaline water (pH = 8 to 8.5), adjusted to pH = 3 and again extracted with EA. This extract is evaporated to dryness *in vacuo*. The luciferin is again dissolved in acid water, adsorbed on celite, and eluted with a butanol-chloroform mixture saturated with water. The fractions containing luciferin are evaporated to dryness and the residue dissolved in acetone. It is crystallized as the free acid, insoluble in water, from a mixture of acetone and water from which the acetone is removed by a current of nitrogen. The crystals of acid luciferin can be removed and washed with water. They dissolve in alkaline water to form the salt of luciferin. Fire-fly luciferin can also be crystal-

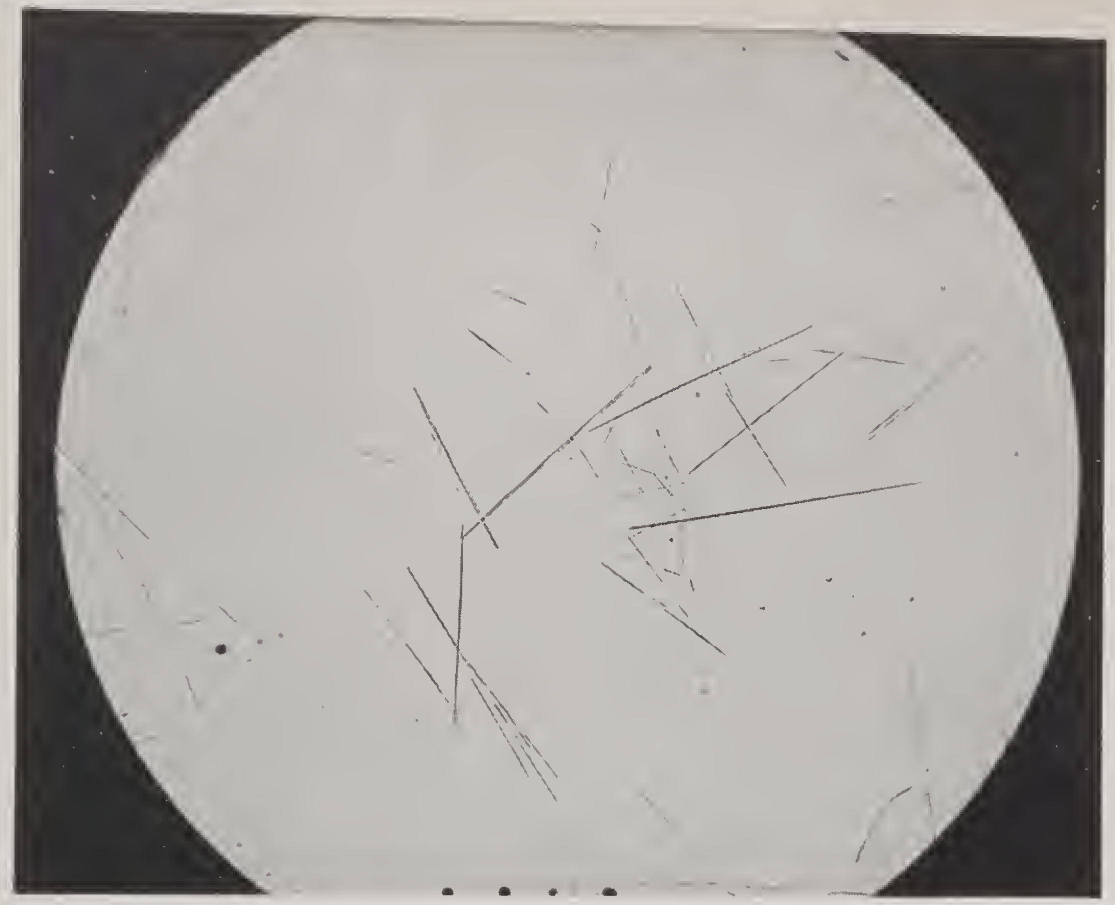

FIG. 4. Crystals of fire-fly luciferase (after Green and McElroy, 78).

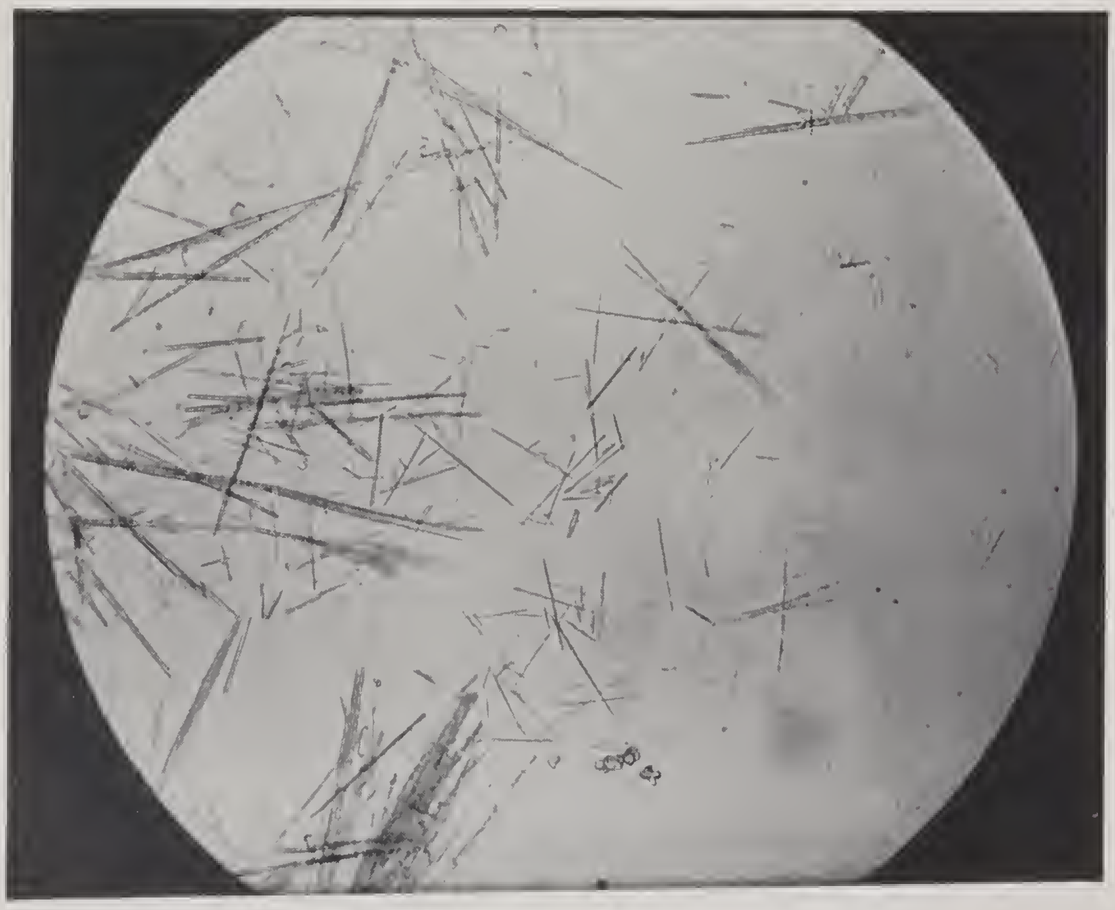

FIG. 5. Crystals of fire-fly luciferin from W. D. McElroy.

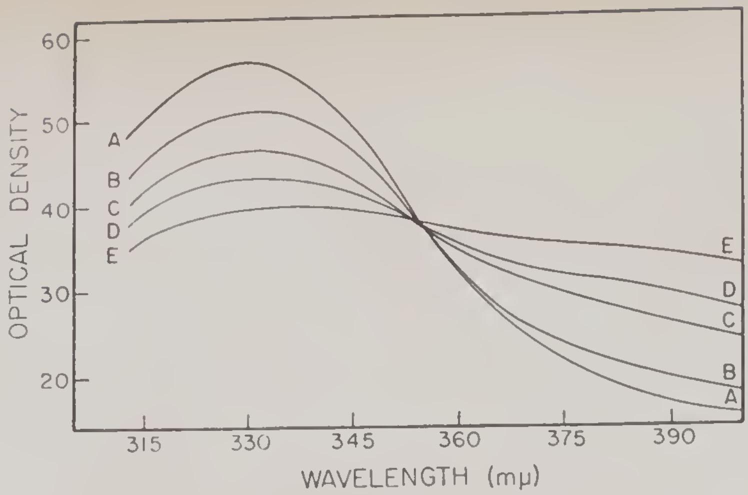

FIG. 6. Spectral absorption changes in fire-fly luciferin during luminescence in a mixture with crystalline luciferase, $MgSO_4$, adenosine pyrophosphate, and inorganic pyrophosphatase at pH 8. Letters give time in minutes and seconds (after McElroy and Green, *74*). KEY: A = 0; B = 25 seconds; C = 2 minutes; D = 3 minutes, 45 seconds; E = 21 minutes.

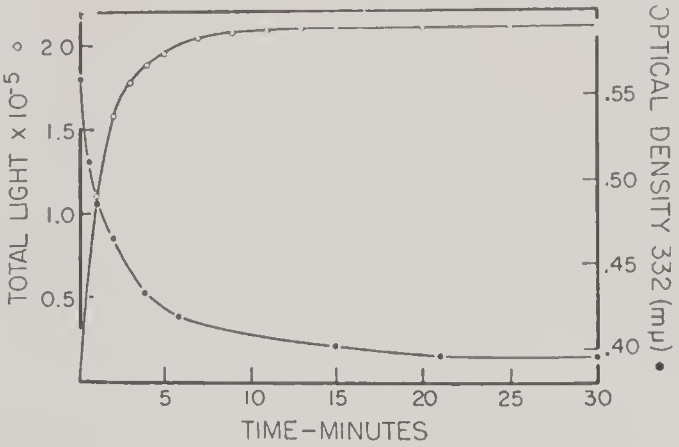

FIG. 7. Optical density changes at 332 mμ in the curves of Fig. 6 above compared with total light emitted at the same time (after McElroy and Green, *74*).

lized as the ammonium salt by passing anhydrous NH_3 through the dry acetone solution. Seventy grams of dry fire-fly lanterns (15,000 fire-flies) yielded 9 mg. of crystalline luciferin.

Elementary analysis of the crystalline material gave the empirical formula $C_{13}H_{12}N_2S_2O_3$, whose molecular weight is 308, while a value of 311 was obtained for the molecular weight by isothermal distillation. Paper chromatography revealed a slight impurity (compound A) with two less hydrogen atoms, which turned out to be oxyluciferin.

Fire-fly luciferin has an ultraviolet absorption maximum at 327 mμ at pH = 6.5. In alkaline solution (pH = 11.8) the absorption peak shifts to 395 mμ and there is some absorption in the blue, making an alkaline solution yellow in color, while the neutral solution is colorless. Both the 327 mμ and the 395 mμ absorption peaks give rise to a marked fluorescence emission (maximum at 530 mμ in neutral and alkaline solution). When mixed with ATP, Mg, luciferase, and oxygen, the chemiluminescent maximum is at 550 mμ. Figure 6 shows the ultraviolet absorption changes in luciferin and Fig. 7 shows how this change at 332 mμ follows the total light emitted during utilization of luciferin.

Luciferin has two pK values, one near pH 3, connected with the COOH group and another at 8.4, probably connected with a phenolic OH. Spot tests show no evidence of aldehyde or ketone, and the sulfur is probably not present as a thioketone or a sulfhydryl group but in a ring. In acid solution luciferin is destroyed rather quickly with formation of fluorescent fragments. It is remarkably stable against oxidation when alone in water.

Fire-fly oxyluciferin undergoes the same change from colorless as an acid to a yellow color in alkaline (salt) condition; the absorption peaks are at 348 mμ in acid (pH 6.5) and 400 mμ in alkaline ($\text{pH} = 11.8$) solution. They also give rise to fluorescence emission peaks, which are at 540 mμ in both acid and alkaline condition.

The adenylluciferin and adenyloxyluciferin have not yet been studied carefully enough for a statement on properties.

Like other organic compounds, fire-fly luciferin changes in solubility depending on whether it is present as free acid or salt, as indicated in the isolation procedure. In the fire-fly it is mostly present as a salt, soluble in water, in all the lower monohydric alcohols, including amyl alcohol, and in ethyl acetate. It is insoluble in benzene, ethyl ether, petroleum ether, chloroform, carbon tetrachloride, and very slightly soluble in acetone. The free acid of luciferin is soluble in acetone, insoluble in water, methanol, ethanol, and petroleum ether, but a complete study of solubilities has not been made.

A very small amount of fire-fly luciferin has been found in nonluminous parts of the fire-fly, but not in other nonluminous beetles tested (*80*).

4. *The Flash*

The final problem connected with fire-fly luminescence has to do with the mechanism of the flash of the living fire-fly. One theory has supposed that this sudden burst of light is connected with the admission of oxygen to the photogenic cells by way of the tracheal system. It has been pointed out by Buck (*81*) that all lampyrids which can flash, i.e., those in which the burst of light lasts of the order of 0.1 second, possess tracheal end cells which may have something to do with the admission of oxygen. Nonflashing lampyrids like the glowworm lack tracheal end cells. The relation of luminescence intensity to oxygen concentration in the fire-fly luminescent system *in vitro* has been studied by Hastings *et al.* (*82*) and the whole problem of the fire-fly flash *in vivo* investigated by Snell (*83*), Alexander (*84*) and Hastings and Buck (*85*).

When fire-flies are kept in absence of air and then air suddenly admitted, they exhibit a pseudoflash, i.e., a burst of light, which lasts a second or two, as first observed by Snell (*83*). The pseudoflash can be initiated in an oxygen-free mixture of luciferin, luciferase, ATP, and Mg in solution, when oxygen is admitted (*82*). The time relations of the burst of light are about the same as the pseudoflash of the intact fire-fly lantern. It has not been possible to accelerate this reaction with oxygen to the point where the flash duration is comparable to that of the normal fire-fly flash; hence McElroy and Hastings (*86*) have concluded that the normal flash of the fire-fly is not controlled by admission of oxygen. The pseudoflash is certainly not due to any activity of the tracheal end cells as it can be obtained from *Pyrophorus,* from glow-worms, and from pupae of lampyrids in which tracheal end cells are lacking, as shown by Hastings and Buck (*85*).

There is another way of initiating the fire-fly flash *in vitro.* When luciferin and ATP solutins are mixed in the presence of luciferase, Mg, and oxygen, the initial light intensity rapidly falls to a low base level due to the reversible inactivation of luciferase by the adenyloxyluciferin. However the addition of pyrophosphate (POP) to this reaction mixture will result in a second flash of rapid rise (0.1 second) and a fall time depending on the concentration of pyrophosphate. This "POP flash" is rapid enough to account for the rapid fire-fly flash *in vivo,* and is believed by McElroy and Hastings (*86*) to explain the normal flash of a fire-fly. The POP splits the inactive complex of luciferase with adenyloxyluciferin, allowing the rapid formation of active luciferin in the cells. POP is thought to be liberated by acetylcholine from nerve endings on the photogenic cells in the following way:

Acetic acid (Ac) from splitting of acetylcholine by cholinesterase liberates pyrophosphate from ATP

$$\mathrm{Ac + ATP \rightleftharpoons Ac{\cdot}AMP + POP}$$

with subsequent liberation of AMP by coenzyme A (CoA)

$$\mathrm{Ac{\cdot}AMP + CoA \rightleftharpoons CoA{\cdot}Ac + AMP}$$

This last reaction forms the acetylating agent (Co·Ac) to restore the acetylcholine in the nerve endings by means of the enzyme choline acetylase:

$$\mathrm{CoA{\cdot}Ac + Choline \rightarrow Acetylcholine + CoA}$$

The POP would be quickly removed by the pyrophosphatase in the lanterns, going to phosphoric acid.

The photogenic cells of flashing fire-flies are large granular cells with several nuclei or a single nucleus, and with ill-defined boundaries. A single cell is supplied by many tracheoles and tracheal end cells. Alexander (*84*) has made the important observation that light appears at points locally at the bases of the tracheoles entering the photogenic cell by way of the tracheal end cells. When a nerve is stimulated these light points do not appear synchronously in any one photogenic cell, but they light independently. In all probability the light appears around the nerve endings as a result of the chemical reactions outlined above. In this respect a fire-fly photogenic cell differs from a multinucleate striated muscle cell, where a single motor-end plate starts a chemical reaction which involves the whole muscle cell. Nevertheless stimulation of fire-fly light organs exhibits many similarities to stimulation of muscle (*87*). By the above chemical changes, it is possible to account for the rapid chemiluminescent flash of a fire-fly.

C. LUMINOUS BACTERIA

1. *Chemical Reactions*

For many years luminous bacteria have resisted the attempts to demonstrate a luciferin-luciferase reaction and in fact to demonstrate the extraction of any compounds which would luminesce *in vitro*. It was chiefly the availability of special pure biological compounds important in cell respiration, such as reduced diphosphopyridine nucleotide (DPNH) and flavin mononucleotide (FMN), as well as the development of very sensitive light-measuring devices, allowing a systematic study of the effect of these compounds on bacterial extracts, which led to an understanding of the several factors involved in bacterial luminescence. It was a well-known fact that luminous bacteria, when quickly dried, would again luminesce for a moment on moistening, and most workers assumed that luciferin and luciferase must be present in these organisms.

In 1953 two groups of workers* almost simultaneously prepared cell-free extracts of luminous bacteria which would emit light on addition of certain substances. Strehler (*89*) and Strehler and Cormier (*89*) used moist luminous bacteria at zero degrees added to acetone at —15° and then dried, in order to prepare an acetone powder, which was homogenized with water and filtered. The yellowish filtrate would give a slowly rising luminescence with diphosphopyridine nucleotide (DPN) and a rapid luminous flash with the reduced form (DPNH). The intensity of the luminescences was affected by other added materials, particularly a factor in an extract of kidney cortex (KCF). Concentrated bacterial extracts always gave best results in these experiments. The work of the Strehler group has been summarized in "The Luminescence of Biological Systems" (*90*).

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* For history see McElroy and Strehler (*88*).

The method of extraction used by McElroy and collaborators (*91*) was the simple cytolysis of marine luminous bacteria in water at low temperature, with removal of the debris by centrifugation. An active preparation which emits light on addition of DPNH was obtained by precipitation of the water extract with HCl at $pH = 4.5$. Further purification was carried out by ammonium sulphate fractionation, but the enzyme (luciferase) lost considerable activity. This activity could be restored by adding flavin mononucleotide, but not by riboflavin or flavin adenine dinucleotide (FAD). McElroy and collaborators (*91*) also found that after the light disappeared in presence of FMN and DPNH, addition of boiled extracts of luminous bacteria would restore the light, and by analogy with the classical luciferin-luciferase reaction designated this unknown compound luciferin. Actually the compound has turned out to be a material similar to that in Strehler's kidney cortex extracts.

This kidney cortex factor (KCF) was subsequently shown by Cormier and Strehler (*92*) to be palmitic aldehyde, and regarded (*93*) as a cofactor in the light-emitting system. It was then shown (*94*) that reduced flavin mononucleotide or reduced riboflavin added to Strehler's cell-free bacterial extracts would luminesce as a flash, and that the light was much more intense if a long chain aldehyde was also added. In Strehler's crude extracts there was in all probability some FMN already present and also some long chain aldehyde, enough to support a low-intensity flash. The reduced riboflavin probably reduced some of the FMN already present, thus accounting for the low-intensity flash when riboflavin was added. McElroy's (*91*) experiments with purified bacterial extracts have indicated that riboflavin and flavin adenine dinucleotide not only fail to take the place of FMN but are potent inhibitors of the luminescent reaction.

Thus, from a complication of factors which influence luminescence in cell-free bacterial extracts it is now clear that the fundamental light-emitting system is made up of luciferase, $FMNH_2$, a long chain aldehyde ($CH_3 \ldots CHO$) and oxygen, all in solution in water. The reactions may be written:

$$FMNH_2 + CH_3.\ .\ .\ .CHO \rightarrow FMNH_2\text{-}CH_3.\ .\ .\ .CHO \text{ (a complex)}$$

This complex may be called bacterial luciferin.

$$FMNH_2\text{-}CH_3.\ .\ .\ .CHO + O_2 \rightarrow FMN + CH_3.\ .\ .\ .COOH + H_2O$$

The flavin mononucleotide can then be reduced for further luminescence.

$$FMN + DPNH + H^+ \rightarrow DPN + FMNH_2$$

The aldehyde and the FMN are assumed to form a complex in order to account for the fact that FMN fluorescence is in the yellow-green, whereas bacterial bioluminescence has a maximum in the blue region of the spectrum.† Such a complexing could occur at the number two carbon of the isoalloxazine ring, in enol form, by removal of water from an aldehyde hydrate. However, such a combination has not been accomplished in the laboratory, either with or without added enzymes.

It is very probable that the aldehyde-flavin mononucleotide complex (luciferin) undergoes a peroxidation, in order to release enough energy for emission in the blue region of the spectrum. In this process the aldehyde is used up, a fact which has been experimentally established. The FMN is then available for reduction and reutilization in the bacterium. The type of flash which appears when FMN is added to partially purified luciferase solution, containing aldehyde, DPNH, and oxygen is shown in Fig. 8. Reduction of FMN occurs by means of DPNH which undergoes oxidation by DPNH oxidase, with FMN as acceptor. Thus in the living bacterial cell a continuous light can be emitted, connected with steady state conditions involving FMN, DPNH, and aldehyde. Reduced triphosphopyridine nucleotide (TPNH) can take the place of DPNH.

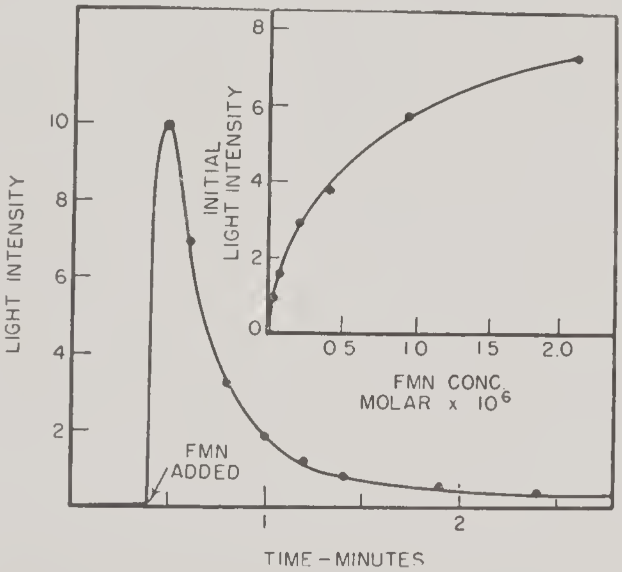

FIG. 8. Luminescence in cell-free bacterial extracts containing luciferase and DPNH-oxidizing enzymes, long-chain aldehyde, and DPNH when flavin mononucleotide (FMN) is added. The light results from reduction of FMN by DPNH, and subsequent oxidation with luminescence (after McElroy *et al.*).

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† Lumichrome derived from lumiflavin has a blue fluorescence.

The evidence that the FMN-reducing enzyme (DPNH oxidase) is different from luciferase is based on the fact that Totter and Cormier (*95*) can destroy the reducing ability of bacterial extracts (due to DPNH oxidase) without affecting the light-producing (luciferase) ability. They also found that a DPNH oxidase from *Escherichia coli* could take the place of the luminous bacterial DPNH oxidase. However, McElroy (private communication) has been unable to separate the two activities by chemical means, and it is possible that only one enzyme is involved. Some of the factors affecting cell-free extract luminescence of bacteria have been reported by Strehler and Cormier (*96*).

Studies of McElroy and Green (*97*) have shown that a plot of the reciprocal of the initial light intensity (I) versus the reciprocal of the square of the $FMNH_2$ concentration gives a straight line, indicating that two molecules of $FMNH_2$ are involved in luminescence. On the other hand $1/I$ versus the first power of the aldehyde concentration gives a straight line, indicating that only one molecule of aldehyde undergoes a change in the light-emitting reaction. The total light emitted is proportional to aldehyde concentration and the aldehyde is only used up under conditions when light is emitted. There is also evidence that $FMNH_2$ undergoes some autoxidation without light emission.

For sufficient energy to account for blue light of 60,000 cal., it would be necessary to assume the peroxidation of an aldehyde, which would yield about 75,000 cal., whereas mere conversion of aldehyde to acid yields only 35,000 cal., and conversion of $FMNH_2$ to FMN about 23,000 cal. McElroy and Green (*74*) have suggested tentatively that because two molecules of $FMNH_2$ are involved in light emission, one goes for combination with the aldehyde and the second for forming an organic peroxide which acts as an oxidant. The energy for blue bacterial light (peak at 480 $m\mu$) would come from peroxidation of the aldehyde to form water and acid as final decomposition products. A study of various long chain aldehydes has indicated that the series will act from C_6 to C_{16}. Palmitic aldehyde was the one found originally in kidney cortex extract. The activity is so great that 5×10^{-7} gm./ml. will result in a visible light in extracts, and blowing the vapor across a culture of nonluminous bacterial mutants (*98*) which lack aldehyde is sufficient to make them luminesce. Too great an aldehyde concentration will cytolyze the bacteria. Rodgers and McElroy (*99*) have studied the penetration rate of the C_6 to C_{14} aliphatic aldehydes into living bacteria, finding that the larger the molecule the slower the penetration rate.

2. *Luciferase*

Since the structure of bacterial luciferin (reduced flavin mononucleotide complexed with long chain aldehyde) can be readily visualized, the question of bacterial luciferase structure becomes of special interest. Separation of luciferase activity from DPNH oxidase activity has already been discussed. Purification has been undertaken by McElroy and collaborators (*91*, *100*). The liquid obtained by water lysis of *Achromobacter fischeri* (after removal of cell debris by centrifugation) is precipitated with HCl at pH = 4.5, and the resultant active precipitate redissolved in water. Fractionation with $(NH_4)_2SO_4$ at 5° gives an active precipitate with 60–70% $(NH_4)_2SO_4$, which is stable for at least two years in the deep freeze. It is colorless, and withstands dialysis at low temperature against distilled water. After repeated fractionation with $(NH_4)_2SO_4$, the material (over 70% of the total protein) is homogeneous by analytical ultracentrifugation, with a molecular weight of approximately 85,000. It contains three components with slightly different electrophoretic mobilities at pH = 7.6. The pH optimum for the bacterial luciferase system lies at pH = 6.7. According to McElroy and Green (*97*) and Totter and Cormier (*95*), bacterial luciferase is a flavoprotein of the FMN type.

A study of different methods of preparing bacterial luciferase from *Achromobacter fischeri* has been made by Cormier *et al.* (*101*). They found that the DPNH oxidase activity can be destroyed by treatment with acid and hence believe that the only function of the enzyme is light production. Some methods of preparation required that albumin be added to the enzyme but the albumin could be replaced by substances which remove heavy metals in solution. Strong inhibition by *p*-chloromercuribenzoate indicates that luciferase is a sulfhydryl enzyme. Hemin is also a potent inhibitor, probably forming a hemoprotein.

Previously the Michaelis-Menton constants (*102*) and kinetic behavior (*96*) of bacterial luciferase preparation from various bacteria had been tested, and their activation energies presented. Some differences have appeared.

Recently the quantum efficiencies of various bacterial enzyme luminescent systems have been investigated by Cormier and Totter (*103*) and the following number of molecules utilized per quantum emitted obtained: FMN, 0.28–0.34; DPNH, 2800; dodecyl aldehyde, 19–43. It has been shown by Strehler and Johnson (*104*) that the continuously luminescing mixture of FMN, DPNH, luciferase, decaldehyde, and oxygen is affected by hydrostatic pressure and temperature in the same way as are suspensions of the living bacteria themselves. The essential facts are illustrated in Fig. 9. These workers also showed that sulfanilamide inhibition of the system *in vitro* was the same as for bacterial cells in that it is scarcely affected by pressure but decreases with rise in temperature. Alcohol inhibition of extracts is decreased by pressure and increased by rise in temperature, as is the case in living luminous bacterial cells. Luminescent oxidation of FMN in presence of aldehyde proceeds with a small volume decrease of activation, whereas reduction of FMN by DPNH proceeds with a large volume increase of activation. The extensive information obtained by F. H. Johnson and collaborators on the effects of drugs, temperature, and pressure on living bacteria will be found in the book of Johnson *et al.* (5, see pp. 187–514).

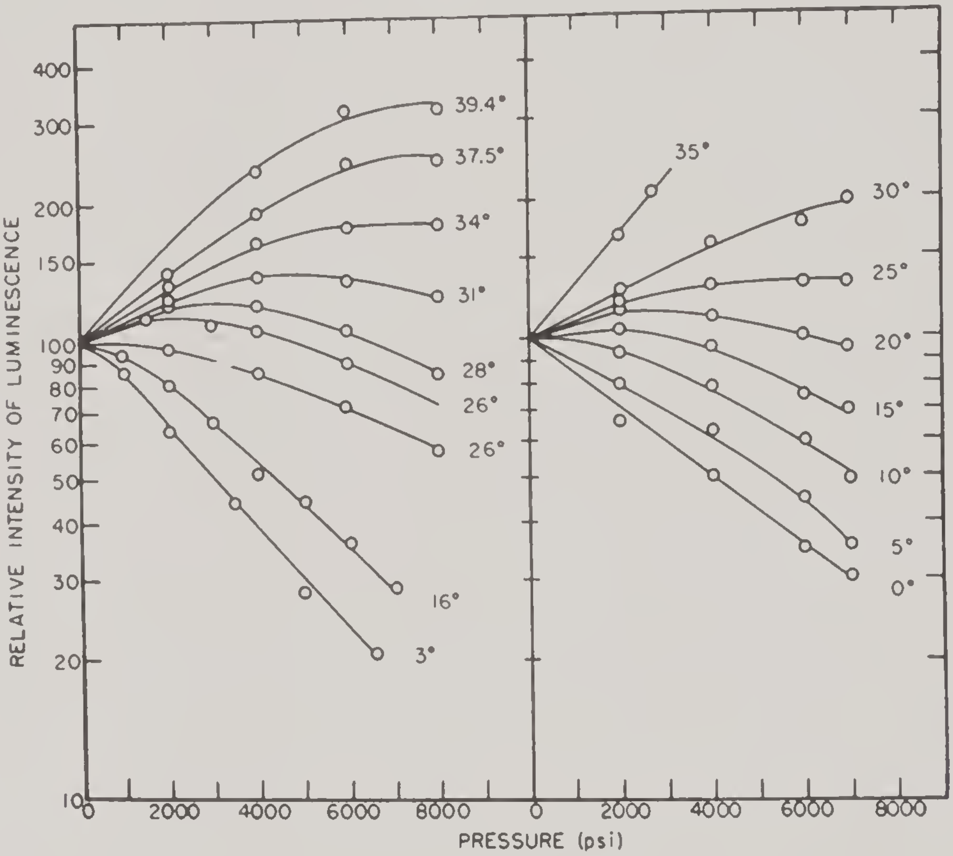

FIG. 9. Left, the effect of hydrostatic pressure in pounds per square inch on the steady state luminescence of cell-free extracts of *Achromobacter fischeri*, containing all necessary constituents for a lasting luminescence at different temperatures. Right, the same for living cells of *Photobacterium phosphoreum*. The initial luminescence at each temperature is taken as 100. Note essential similarity of the pressure-temperature effects. The lower of the two curves at 26° was recorded after several days (after Strehler and Johnson, *104*).

Just as a bacterial suspension when deprived of oxygen will give a flash of excess luminescence before returning to steady state luminescence intensity when air is readmitted (*105*, *106*) so anoxic mixtures of FMN + aldehyde, DPNH, and luciferase will give an excess flash when air is admitted (*107*). The excess luminescence is due to accumulation of $FMNH_2$ under anaerobic conditions, the same phenomenon as observed in bacterial suspensions. Hastings (*108*) has studied oxygen pressure and luminescence intensity in both bacteria and in extracts, finding the same relations.

V. Other Luminescent Systems

A perusal of Table I will indicate that the luciferin-luciferase reaction has been demonstrated in four, possibly five, other groups besides the luminous beetles, the bacteria, and the ostracods.† These are certain deep-sea decapod crustacea (*Systellaspis*) which eject a copious secretion into the sea water (*109*), the syllid worms (*109*), the fresh water limpet, *Latia* (*110*), and the bivalve mollusc, *Pholas,* first described by Dubois (*111*). Some authors have described a luciferin-luciferase reaction in earthworms and others have failed. Nothing further is known of the chemistry of the decapod crustacea, the syllid worms, or the limpet, except that luciferin from *Cypridina* will not give light with luciferase from any of these three forms, or with *Pholas* luciferase. The converse is also true; *Cypridina* luciferase will not react with luciferin from any of the above organisms. An extended study by the author (*109, 112*) over many years has indicated that only if animals are very closely related (allied genera or perhaps families), will luciferin from a species of one genus or family give light with luciferase from a species of another genus or family.† This fact may be taken to indicate that the luminescent systems in various luminous groups may be quite different.

Frequently luminous species are hard to get, deep sea animals, for example. Relative little is known concerning the rare railroad worm, *Phrixothrix,* despite the fact that it emits light of two colors, yellow-green and red. The red luminescence suggests that a porphyrin may be the emitter but it has not been possible to prove this. Table I indicates that the luminous beetles are the principle ones requiring ATP for luminescence, although a final statement on certain other species must await further information.

The luminous organisms, not previously considered in detail, which might be easily obtained in large quantity are fungi, dinoflagellates, hydromedusae, and scyphomedusae, pennatulids, ctenophores, certain marine worms, earthworms, *Pholas,* certain squid (*Watasenia*), copepods, schizopod and decapod shrimp, diplopod and chilopod myriapods, diptera (New Zealand glowworm), ophiuroids, balanoglossids, tunicates

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† Recently Y. Haneda, F. H. Johnson, and E. H. C. Sie (*Biol. Bull.* **115,** 336, 1958) have demonstrated the luciferin-luciferase reaction in a fish, *Apogon marginatus,* and shown that cross reactions with *Cypridina* luciferase and luciferin will occur. Haneda and Johnson have also demonstrated the luciferin-luciferase reaction in the fish, *Parapriacanthus bercyformis,* of the Pemphyridae (*Proc. Natl. Acad. Sci. U. S.* **44,** 127–129, 1958).

(*Pyrosoma*), and some fish. Little is known of their chemistry of luminescence that is not presented in Table I. Exceptions to this statement include the dinoflagellates, the earthworms, and *Pholas.*

The fungi (*32*), the diplopod myriapod, *Luminodesmus* (*113*), and the ctenophores have resisted extraction of luminous material even by use of the most modern methods. Chilopod myriapods present intriguing sources of material, if they could be cultivated in numbers. Scyphomedusae and pennatulids should be studied again. In these groups, and also in ctenophores the luminescence seems to be connected with granules. Luminescence appears when the granule dissolves, a process which has been called granulolysis, induced by the same agents which cause cytolysis of cells. The solution of granules appears to be involved in luminescence of many other species, for example, in earthworms, in myriapods, and in *Pholas.* The process should be further investigated. It is possible that the photogenic granule represents a luminescent system, comparable to the enzyme-cofactor systems present in microsomes or mitochondria. It is obvious that an intensive study of the structure of photogenic granules should be carried out. What is known of *Pholas*, of the earthworm, and of dinoflagellates is presented below.

A. *Pholas*

A modern study of the biochemistry of luminescence in *Pholas* is much to be desired. The author, in unpublished experiments, has confirmed many of the statements of Dubois, including the importance of photogenic granules, the existence of luciferin and luciferase, and the fact that something in the boiled extracts of *Pholas* light organs (probably luciferin) will luminesce on addition of $KMnO_4$ and other oxidizing agents. Dubois (*114*) has listed the properties of the photogenic substances of *Pholas* as follows: *Pholas* luciferin is destroyed above 70°, dialyzes slowly, oxidizes with light production in the presence of *Pholas* luciferase, potassium permanganate, hydrogen peroxide, hematin, and hydrogen peroxide, barium peroxide and lead peroxide, hypochlorites, and the blood of various marine mollusks and crustacea. It is insoluble in fat solvents, but forms a colloidal solution in water from which it is precipitated unchanged by picric acid, alcohol, and ammonium sulphate. It is not precipitated by sodium chloride or acetic or carbonic acids, except in presence of neutral salts. It forms an insoluble "alkali albumin" with ammonium hydroxide which increases the luminescence.* At various times Dubois has regarded luciferin as a proteose, as a nucleoprotein, and as a natural albumin having acid properties. It occurs only in the luminous parts of *Pholas,* not in nonluminous animals.

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* The author is listing the properties as Dubois has described them.

Dubois found that *Pholas* luciferase has all the properties of an enzyme, is destroyed at 60°, is nondialyzable and insoluble in fat solvents, but forms a colloidal solution in water. It is not affected by 1% sodium peroxide but its activity is suspended in saturated salt solutions, sugar, or glycerine, with activity returning on dilution. It is digested by trypsin and slowly destroyed by the fat-solvent anesthetics such as chloroform. Dubois regarded the enzyme as a special kind of oxidase, called it a carrier (emprunter) of oxygen in his latest (*115*) paper. He found luciferase or substances capable of giving light with *Pholas* luciferin in the blood of many nonluminous crustacea and molluscs.

In addition to luciferin and luciferase, Dubois has described a number of other accessory luminous substances. It is obvious that luciferin must be formed from some precursor in the cell and following the usual biochemical terminology, Dubois called this substance proluciferin or preluciferine, and believed that it is converted into luciferin by another enzyme, coluciferase.

It is probable that the luminescent reaction in *Pholas* is complicated by the presence of photogenic granules (vacuolides of Dubois) observed by all workers. Anything which dissolves these granules results in light emission. A sea-water extract of the siphons of *Pholas* allowed to stand until the luminescence disappears will again emit light on dilution with distilled water, on slow heating, or addition of ether, chloroform, saponin, or sodium glycocholate. These are all cytolytic effects and the luminescence is due to solution of granules. These granules can be seen to dissolve under the high power of the microscope if the luminous organs are teased on a slide and a little saponin powder added. The solution of the numerous photogenic granules takes place like a flash and this resulting solution of the granules still contains luciferase, for it will react with *Pholas* luciferin and emit light—the true luciferin-luciferase reaction. Drying the luminous tissue of *Pholas* will also abolish the luminescence due to granulolysis. All attempts to demonstrate interaction of *Pholas* luciferase with *Cypridina* luciferin or *Cypridina* luciferase with *Pholas* luciferin have been negative.

B. EARTHWORMS

As previously pointed out, an extensive study of the luminous earthworm, *Eisenia submontana,* has been made by a number of Czech workers, particularly Wenig (*30*). The yellow-green bioluminescence of this form appears when disturbed, by ejection of lymph from its body cavity through the dorsal pores. The light comes from granula, probably of a lipoprotein nature in the lympocytes. These granula have been described (*116*) as dissolving with light emission. They contain a yellow pigment which Wenig (*30*) showed to have a fluorescent maximum at 550 mμ and to behave in every way like riboflavin. On reduction, the yellow color and the fluorescence disappear. Wenig and Kubišta (*31*) later showed that the yellow pigment was not flavin mononucleotide or flavin adenine dinucleotide. In addition another substance with a blue fluorescence in alkaline reaction is present in the extracts of the lymphocytes. Extracts which have ceased to bioluminesce exhibit a blue fluorescence, and a blue fluorescence is also observed after exposure of the extracts to ultraviolet light; this is the same change which riboflavin undergoes in neutral or weakly acid media by conversion to lumichrome. If the extract is made alkaline and exposed to light, a chloroform soluble substance is formed with the properties of "lumilactoflavin." Molecular oxygen is necessary for bioluminescence, and cannot be replaced by other oxidizing agents. The ability of extracts to luminesce cannot be regained by addition of nascent hydrogen or pulverized zinc or magnesium, or reduced dyes. Komerek and Wenig (*117*) were able to demonstrate a luciferin-luciferase reaction, although Gilchrist (*116*) failed, using a South African earthworm, *Chilota,* and the author (*112*) obtained negative results with *Microscolex phosphoreus* at Naples. Komerek and Wenig emphasized the importance of granules for luminescence, finding that they emit light when treated with cytolytic agents. Oxidizing agents did not necessarily cause light emission, although H_2O_2 would call forth a long lasting light.

The remarkable aspect of *Eisenia* luminescence is the fact that another closely allied species to *E. montana,* namely *E. foetida,* is nonluminous and yet contains the same yellow granules in its lymphocytes. The yellow color is again due to riboflavin and not flavin mononucleotide or flavin adenine dinucleotide (*31*). Bačkovský and co-workers (*118*) attempted to explain the nonluminescence of *E. foetida* to lack of a factor, present in *E. montana,* which converted riboflavin to lumiflavin, which they believed to be responsible for the light emission.

Wenig's final statement (*30*) of the mechanism of light production in the earthworm is as follows:

"Molecules of lactoflavin (riboflavin), adsorbed in an oriented layer on the surface of granula of a lipoid character, are considered as the source of the light. The activation energy which brings them into an excited state is probably derived from an oxidative reaction in which molecular oxygen takes part. The possibility is discussed whether the energy set free is sufficient for the activation of the neighboring molecules of flavin, enabling them to radiate light of the corresponding colour." Unfortunately this discussion is in Czech.

C. Dinoflagellates

With the perfection of methods for growing marine dinoflagellates in culture media in large quantity, it is now possible to describe some of the chemical characteristics of cell-free extracts. Hastings and Sweeney (*119*) have described the method of extraction from an armored dinoflagellate, *Gonyaulax polyhedra,* which contains chlorophyll and only lives for any length of time if occasionally illuminated. This organism exhibits a day-night rhythm of luminescence (on stimulation), which persists at least 14 days in continual dim light and for several days in darkness, before the organism dies from lack of photosynthetic food. In bright light there is practically no luminescence on stimulation, even during periods which correspond to night. There is also a periodicity of cell divisions—most of them occurring toward the end of the night period—a rhythm which persists in continued darkness and in continued dim light (*120*). During the process of cell division in cultures, there can be recorded a continuous dim glow, independent of stimulation (*120*).

Corresponding to the day-night rhythms of luminescence, it has been found (*119*) that five to fifteen times more luminescence can be observed in crude extracts prepared at midnight than in those prepared at noon. On the other hand, the living organisms are 40 to 60 times brighter at night than in the dark in daytime.

Crude extracts are made by collecting the organisms from the culture on filter paper on a Buchner funnel, washing with distilled water to remove salts, and plunging the mass into acetone at —25°. The acetone is removed by filtration and evaporation. This "acetone powder" gives a faint light on adding water and a much brighter light with 1.2 *M* salt. The maximum light emission for this crude extract and also of purified preparations is at 478 mμ, the pH optimum is at 6.6, and the temperature optimum at 24°. Oxygen is required for luminescence, but no excess luminescence is observed after a period of anoxia, when air is readmitted. The enzyme luciferase in the crude extracts has been partially purified by fractional precipitation with $(NH_4)_2SO_4$. Most of the activity comes down between 30 to 60% saturation, with a maximum precipitation at 40 to 50%. On dissolving the precipitate in water, a solution is obtained which will luminesce with a boiled crude extract of *Gonyaulax.* This factor may be called "luciferin." Further analyses of the factor has not been made, as attempts to extract a possible luciferin from acetone powder with a variety of solvents (alcohols, ethyl acetate, etc.) has failed. "Luciferin" dialyzes slowly but dialysis does not appear to be a promising method of separation. The result of adding various cofactors known to be effective in other luminescent systems (ATP, DPNH, flavins, etc.) has been tested with luminescing *Gonyaulax* extracts but no active substance discovered. Nothing can be stated concerning the chemical nature of "luciferin," but the system appears to be simple, like that of *Cypridina*. However, *Cypridina* luciferin will not luminesce when added to crude or purified extracts of *Gonyaulax*.

Although there is the above evidence that a luciferin exists, it must be noted that adding boiled (then cooled) crude luminescent extract of *Gonyaulax* to the crude extract whose luminescence has ceased will not restore the light (the classical luciferin-luciferase reaction). Only if the luciferase has been purified by $(NH_4)_2SO_4$ precipitation will the boiled extract restore luminescence. This result is probably due to the presence in the crude extract of an inhibitor, removed by the purification procedure. This situation is known to exist in fire-fly luminescence.

VI. Evolution of Bioluminescence

There are always two aspects to any evolutionary problem: (1) the first beginnings of a new organ, in this case the appearance of chemical reactions which emit light; (2) the further evolution of accessory structures, in this case the development of lenses, reflectors, and pigment screens. The perfected organ forms a lantern to direct the light, such as are found in many deep-sea organisms. The comparative biochemistry of luminescence is only concerned with the first aspect of the problem. The author (*121, 122*) has emphasized this point in previous reviews of the evolution of bioluminescence.

It is apparent from the foregoing discussion that the substances in the luminescent system of bacteria, the simplest light-emitting organisms, are practically identical with the system necessary for respiratory oxidations in various cells. The author (*105*) predicted that the evolution of light production would turn out to be of this nature as long ago as 1932, and it seems likely that the structure of fire-fly luciferin and *Cypridina* luciferin will not be very different from well-known substances of a respiratory pathway.

It is common observation that in many orders of organisms are found two varieties or species, morphologically closely related, one of which is luminous and the other not. The clearest examples occur among luminous bacteria, where nonluminous strains or mutants have been known for a long time. It was Beijerinck (*123*) who first realized the significance of this fact in a paper, "On Different Forms of Hereditary Variation of Microbes." His views were extended in later papers (*124, 125*). All workers with luminous bacteria have noted the dim and dark mutants which Beijerinck described and a number of persons have made use of them in genetic studies. A most important investigation was carried out by Doudoroff (*126*) who endeavored to find out what constituent of the culture medium was responsible for the loss of luminescence. He discovered that with some of his dim strains, but not with all, the addition of "lactoflavin" to the culture medium would restore the luminescence intensity, and concluded that flavin or a derivative of flavin was connected with one of the enzymes involved in production of light by the bacteria.

Giese (*127*) described the converse of a dark mutant, a "brilliant variant," which appeared when the medium was allowed to become acid, although the new variant would grow on calcium carbonate buffered agar. This mutant developed a yellow pigment, which diffused into the medium and would fluoresce in ultraviolet light, thereby suggesting riboflavin.

Probably the most interesting mutant has recently been described by Rodgers and McElroy (*128*). It is one which contains all the essential substances for luminescence except the long-chain aldehyde. Rodgers and McElroy found that addition of minute amounts of dodecaldehyde to colonies of this dark mutant immediately restored the light. They also proved that the dark aldehydeless mutants contained bacterial luciferase which could be extracted and would act *in vitro* in the absence of bacterial cells, provided long-chain aldehyde, FMN, and DPNH were present. Presumably an aldehyde-forming or aldehyde-releasing enzyme is lacking in this particular mutant.

These authors also observed other dark mutants, which could not be made to luminesce by addition of FMN, DPNH, or long-chain aldehyde, i.e., by any of the cofactors involved in bacterial luminescence. Rodgers and McElroy suggest that in such a case luciferase may be blocked or lacking. As a result of the above studies, luminous bacterial mutants lacking luciferin, or aldehyde, or luciferase are now available.

Since the ability to luminescence has appeared in bacteria of several different morphological types (rod-shaped forms, cocci, and vibrios), involving chemical systems of widespread occurrence in living cells (flavin mononucleotide, flavoprotein enzymes, and aldehydes), might not a luminous mutant suddenly appear in a well-known and much-studied form such as *Escherichia coli*? If phage-resistant or antibiotic-resistant mutants are found in nature, why not luminous mutants of common species also? Perhaps the innumerable strains of luminous bacteria which have been described are actually luminous mutants of certain species which normally are nonluminous. The possibility has not been properly tested. It would be worth while to find out if the nonluminous saprophytic bacteria which can be isolated from the skin of marine fish really represent the "normal" bacterium. They are discarded because the investigator has set out to isolate the more striking luminous mutant. Certain it is that the luminous *Vibrio albensis,* obtained from the Elbe River by Sonnenschein (*129*) is very closely allied to the cholera *Vibrio,* with similar immunological reactions and similar relations to attack by bacteriophage. Perhaps the goal of the investigator in this field should be the development of a luminous strain of yeast, or a luminous ameba.

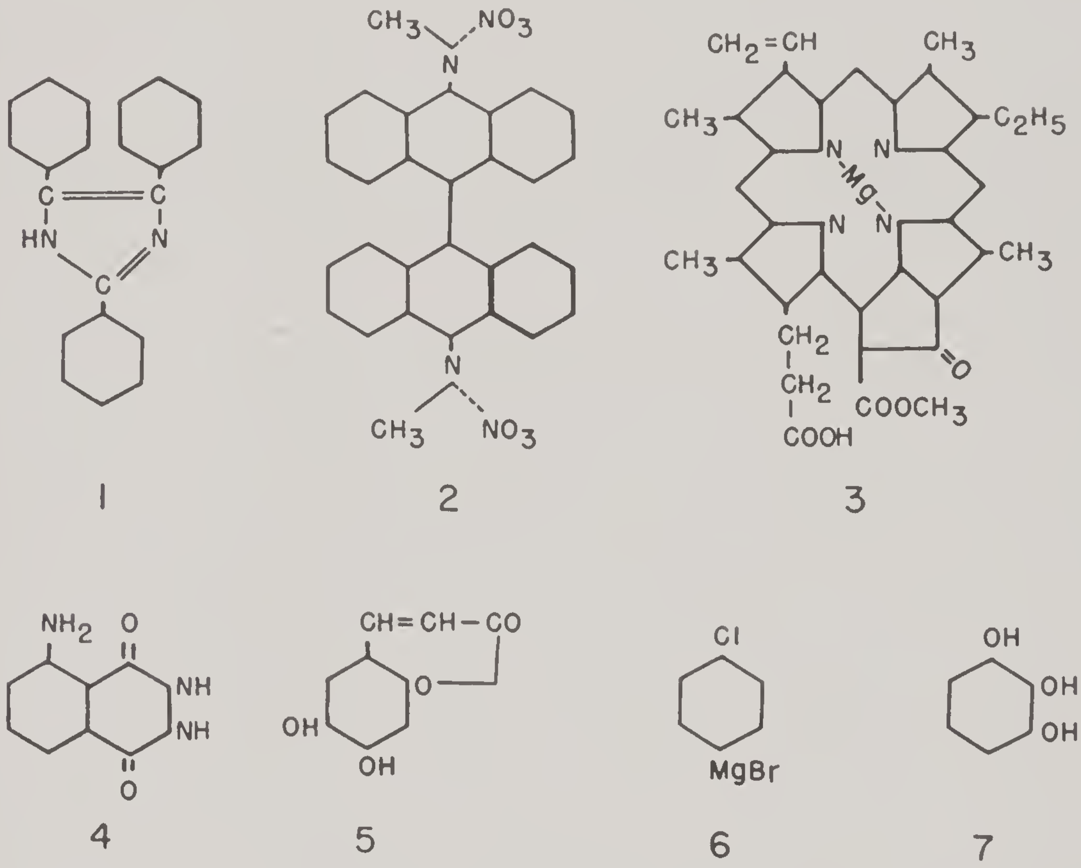

FIG. 10. Various types of organic chemiluminescent compounds: 1, lophine or triphenylglyoxaline; 2, dimethyldiacridinium nitrate; 3, a metal porphyrin compound; 4, luminol or aminophthalichydrazide; 5, aesculetin, present in the glucoside, aesculin; 6, chlorophenylmagnesium bromide; 7, pyrogallol.

Thus the origin of luminescence in simple cells like bacteria is no different from the origin of other characteristics, for example, colors or fluorescent substances. Just as a slight change in the molecule can result in the formation of a brilliant pigment, so a similar change can produce a chemiluminescent compound. The living cell has many possibilities to choose from, as indicated in Fig. 10, which shows the wide variety of structural make-up among organic chemiluminescent compounds.

A concrete case in which a very slight change in a molecule is found to bestow the ability to chemiluminescence occurs among the phenylcyclohydrazides (phthalic hydrazides), studied by Drew and coworkers (*130*). In these compounds the presence of various groups in different positions greatly influences the intensity of the luminescence when catalyzed by hemin or other organic metal-containing compounds. Such catalysts can be compared to luciferase. For example, aminophthalichydrazide with a methyl group substituted in place of a hydrogen of an NH group would be nonchemiluminescent with hemin and hydrogen peroxide, but when the methyl group is removed, the hydrogen containing aminophthalichydrazide becomes brilliantly luminescent. If such a compound were present in the organism, a demethylating enzyme (gene) would result in a luminous mutant.

Removal of the amino group makes aminophthalichydrazide much less chemiluminescent. The appearance of a deaminase in an organism might result in a dim mutant. It is by such small changes as this that the first beginnings of luminescence in a cell must have occurred.

Non-luminous varieties are also found in more complicated organisms. Among higher fungi, *Panus stipticus* of Europe is nonluminous but the American variety, *P. stipticus* luminescens, emits light from parts of the fruiting body and the hyphae, after they have grown a certain amount. Other fungi, for example, *Mycena galopus* and *Mycena polygramma,* observed by Bothe (*131*), exist in two varieties luminous and nonluminous. Macrae (*132, 133*) has made extensive genetic studies of the luminous and nonluminous *Panus stipticus* strains, and finds that they are completely interfertile. Diploid mycelia and fruit bodies of the F_1 generation are always luminous. Studies on the haploid mycelia from an F_1 fruit body indicate that emission of light is "governed by a single pair of Mendelian factors in which luminosity is dominant over nonluminosity and that the luminosity factors form all possible combinations with the infertility factors."

We have already seen that in the animal kingdom one species may be luminous and a closely allied species not. The earthworm, *Eisenia montana,* is luminous while *Eisenia foetida* is not. Some species of the dinoflagellate, *Ceratium,* are luminous, others not, while the famous *Noctiluca,* popularly regarded as the universal cause of "phosphorescence of the sea," may exist in a nonluminous condition.

Similar examples could be multiplied many times. They indicate how the ability to luminesce could have appeared at various periods of evolution and in widely separated phyla of the animal kingdom. The "first beginnings" of luminosity by mutation therefore presents no problem, but it must be admitted that the factors involved in the change of a luminous spot to a complicated lantern are far from clear.

One of the early steps in the evolution of bioluminescence has been the development of a mechanism for controlling the light. It has been pointed out that control of the light is found throughout the animal kingdom (with a few exceptions), and is usually mediated by nerves. In most of the metazoa, a reflex stimulation of light production is the rule, for example in the flash of the fire-fly. Mechanical stimulation is usually sufficient to initiate the reflex, but in some fish (*1*), such as *Porichthys*, *Echiostoma*, and *Argyropelecus*, light does not readily appear even upon strong mechanical stimulation. However, every photophore becomes brilliantly luminous if a little adrenaline is injected into the fish.

In Protozoa also, light appears only on stimulation, even when the organism contains chlorophyll. The slightest agitation of either *Gonyaulax*, a green dinoflagellate, or the colorless *Noctiluca* is sufficient to elicit a flash. Successive flashes fatigue the organism, and deleterious substances or conditions result in a "death-glow," the constant emission of a weak light.

The response of *Noctiluca* and other luminous flagellates is a typical "stimulus reaction," quite comparable to stimulation of a nerve or muscle cell. The question arises whether electrical phenomena accompany the flash of light. Action potentials have been described during the luminescence of Metazoa, but the origin of these potentials is not certain. In the case of *Noctiluca*, Hisada (*134*) has observed an action potential accompanying tentacle movement, but was not sure of a potential change during luminescence.

The ability to respond to stimuli is one of the characteristics of living things which appeared early in the course of evolution, almost as soon as the aggregation of large molecules to form a cell. The evolution of "stimulus reactions" has not been sufficiently discussed, so intent has the evolutionist been on morphological characteristics, but it is certainly true that response to stimuli by luminescence is found as far down in the evolutionary scale as response to stimuli by movement of one kind or another. The previous discussion of the flash of the fire-fly has indicated a possible chemical mechanism for stimulation of luminescence.

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